



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12Q 1/68	A1	(11) International Publication Number: WO 90/05789 (43) International Publication Date: 31 May 1990 (31.05.90)
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(21) International Application Number: PCT/US89/05253

(22) International Filing Date: 15 November 1989 (15.11.89)

(30) Priority data:
271,609 15 November 1988 (15.11.88) US

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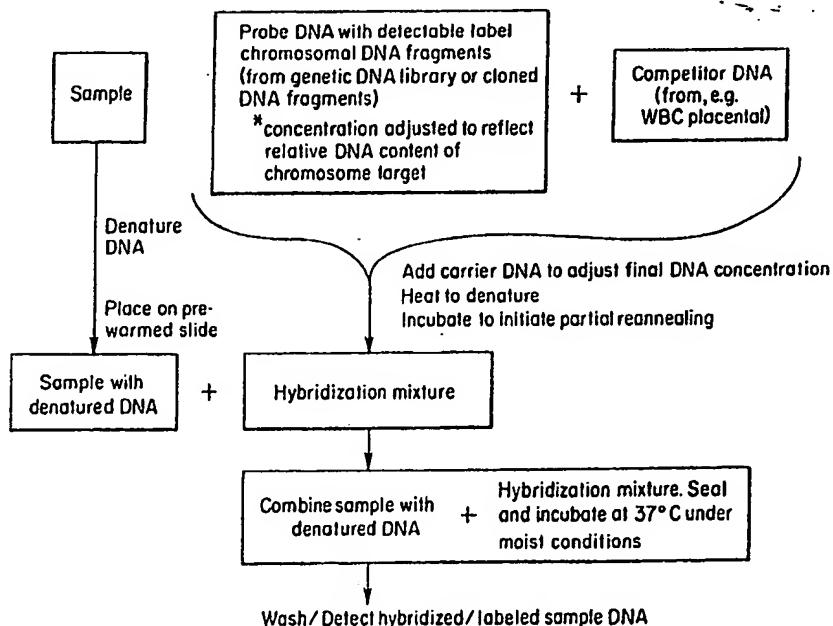
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(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, FI, FR (European patent), GB (European patent), HU, IT (European patent), JP, KR, LU (European patent), NL (European patent), NO, SE (European patent), SU.

Published

With international search report.

(54) Title: IN SITU SUPPRESSION HYBRIDIZATION AND USES THEREFOR

OUTLINE OF CISS HYBRIDIZATION FOR SPECIFIC
STAINING OF HUMAN CHROMOSOMES

(57) Abstract

A method of specifically decorating selected mammalian chromosomes and of detecting, identifying and and/or quantitating selected individual chromosomes, by means of chromosomal in situ suppression (CISS) hybridization. The method is useful in analyzing cells for the occurrence of chromosomes, chromosome fragments or chromosome aberrations.

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IN SITU SUPPRESSION HYBRIDIZATION AND USES THEREFOR

Description05 Background

Chromosome banding techniques have facilitated the identification of specific human chromosomes and presently provide the major basis upon which chromosomal aberrations are diagnosed. The interpretation 10 of chromosome banding patterns requires skilled personnel and is often technically difficult, especially with respect to detecting minor structural changes and when analyzing complex karyotypes, such as those of highly aneuploid tumor cells. An 15 additional complexity is that readable metaphase chromosome spreads are sometimes very difficult or impossible to prepare from certain cell types or tissues. Alternative methods for identifying chromosomal aberrations would be valuable because 20 they could augment current methods of cytogenetic analysis, particularly if such alternative methods were applicable to both mitotic and interphase cell populations.

Over the past few years, a considerable body of 25 evidence has been obtained which indicates that the DNA of individual chromosomes occupy focal territories, or spatially cohesive domains, within mammalian interphase nuclei. Cremer, T. et al., Hum. Genet., 60:46-56 (1982); Hens, L. et al., Exp.

Cell Res., 149:257-269 (1983); Schardin, M. et al., Hum. Genet., 71:281-287 (1985); Manuelliidis, L., Hum. Genet., 71:288-293 (1985); and Pinkel, D. et al., Proc. Natl. Acad. Sci. USA, 83:2934-2938 (1986).

05 These observations suggest that chromosome-specific probe sets could be used to detect numerical or structural aberrations of chromosomal domains in non-mitotic cells, an approach termed "interphase cytogenetics". Cremer, T. et al., Hum. Genet., 74:346-352 (1986). Indeed, recent *in situ* hybridization studies have demonstrated the prenatal diagnosis of trisomy-18 with interphase cells and the detection of numerical chromosomal abnormalities in tumor cells lines using chromosome-specific repetitive DNAs as probes. Cremer, T. et al., Hum. Genet., 74:346-352 (1986) and Cremer, T. et al., Exp. Cell Res., 176:119-220 (1988). All chromosome-specific repetitive DNAs reported to date are localized to discrete subregions of each chromosome and, thus, such DNA probes are unsuitable for analyses of many types of chromosomal aberrations (e.g., translocations and deletions). If it were possible to detect uniquely the spectrum of sequences comprising a specific chromosome, analysis of aberrations of chromosomal domains in non-mitotic cells would be possible. Furthermore, such a general labeling technique would make it possible to address fundamental questions concerning the spatial organization of chromosomal DNA within interphase nuclei.

Disclosure of the Invention

The subject invention relates to a method of detecting, identifying and/or quantitating selected individual chromosomes in mammalian mitotic or

05 interphase cells, by means of chromosomal *in situ* suppression (CISS) hybridization and its use in analyzing cells for the occurrence of chromosomes, chromosome fragments, or chromosome aberrations, such as those associated with a condition or

10 disease. In the method of the present invention, chromosome-specific probes (DNA or RNA) are combined with a sample to be analyzed, in such a manner that an individual chromosome(s) of interest is labeled and the complex spectrum of sequences which comprise

15 the chromosome can be detected. The probes used in the present method are of high genetic complexity and can be appropriately-selected cloned DNA or RNA fragments, used individually or in pools, or chromosome library DNA.

20 The method of the present invention, referred to as CISS hybridization, is particularly useful because it can be used to specifically stain individual mammalian chromosomes at any point in the cell cycle. It can be used to assess chromosomal

25 content, particularly chromosome aberrations (e.g., deletions, rearrangements, change in chromosome number) which, until the present invention, it has been time-consuming and/or difficult, if not impossible, to detect. The method is useful in

30 providing a rapid and highly specific assessment of individual mammalian chromosomes in any context

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(e.g., diagnosis and/or monitoring of a genetic condition or a disease state) in which such an assessment is desired.

Brief Description of the Drawings

05 Figure 1 presents an outline of the CISS hybridization method of the present invention.

Figure 2 shows suppression from cross-reacting sequences within a chromosome 7-derived DNA library by different concentrations of human competitor DNA.

10 Biotin-labeled chromosome 7 DNA inserts (20 μ g/ml) were prehybridized for 20 minutes with human genomic DNA prior to hybridization with metaphase chromosome spreads and detection with FITC-labeled avidin.

15 Human DNA concentrations were: A, 0 μ g/ml; B, 50 μ g/ml; C, 100 μ g/ml; D, 200 μ g/ml; E, 1000 μ g/ml; F, same metaphase spread as in E post-stained with DAPI. Genomic salmon DNA was added to each sample to adjust the final DNA concentration to 1.0 mg/ml (see the text for details). The arrows mark the

20 target chromosome 7 and the arrowheads mark additional strong signals on non-7 chromosomes. All negatives printed were exposed and developed under identical photographic conditions.

Figure 3 shows the effect of pre-annealing time 25 on the specificity and strength of the hybridization signal. Biotin-labeled chromosome 7 DNA inserts (20 μ g/ml) were preannealed with 200 μ g/ml human competitor DNA for the following times, prior to hybridization to metaphase A, 0 minutes; B, 2 30 minutes; C, 5 minutes; D, 20 minutes.

Figure 4 shows decoration of A chromosome 1, B chromosome 7, C chromosome 4, D chromosome 18, E chromosome 13 and F chromosome 20 in normal human lymphocytes. Only the chromosome 13 insert DNA pool 05 shows significant cross-hybridization to other chromosomes after the prehybridization suppression step. The detection of chromosome 20 (F) was done with the entire chromosome library (including λ phage arms) and detected with avidin-alkaline 10 phosphatase using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT-BCIP) as the enzyme substrate mixture. The signal of chromosome 1 (A) was amplified by the sandwich technique of Pinkel et al. (1986).

15 Figure 5 shows chromosome domains in human lymphocyte nuclei delineated by preannealed chromosome library DNA inserts. Hybridization to acetic acid-methanol fixed nuclei was detected by fluorescein isothiocyanate (FITC)-conjugated avidin 20 (A-E) or alkaline phosphatase-conjugated avidin (F). Domains are shown for chromosome 1 (A,B), chromosome 7 (C,D) and chromosome 18 (E,F). A predominant staining of the centromere region is seen within the chromosome 7 domains, reflecting preferential 25 hybridization of the chromosome 7-specific alphoid DNA repeat; a similar signal distribution on metaphase chromosomes was also observed in this particulate experiment.

30 Figure 6 shows chromosomal *in situ* suppression (CISS) hybridization of chromosome 1 inserts to metaphase spreads of glioma cell lines detected with

avidin-FITC (A,C) and poststained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) (B,D). A,B TC 620 show two apparently complete 1 chromosomes (small arrows in B) and two marker 05 translocation chromosomes (arrowheads) specifically decorated by these inserts (A). One of the two marker chromosomes contains a 1p (lower left), the other a 1q arm (lower right); the 1p terminal (relatively GC rich region) in the two normal 10 chromosomes and submetacentric marker is less completely delineated. Also, the 1q12 regions here show little decoration in contrast to most experiments. X 950. C,D Typical TC 593 metaphase spreads show six specifically decorated chromosomes. 15 Three acrocentric marker chromosomes all with truncation of 1p show particularly intense fluorescence of repeats that localize to 1q12 (arrows in C). In two of these, 1q arms appear to be complete, while a major deletion is obvious in 20 the third (the arrow in D). A fourth decorated chromosome (small arrowhead in C,D) again shows a major deletion of the distal part of 1q, but has retained an apparently complete 1p arm. A fifth submetacentric chromosome (large arrowhead) contains 25 an apparently complete 1p arm; the DNA of its short arm has not been identified. Note the similarity of this marker to one of the marker chromosomes of TC 620 (1p) described above. The sixth entirely decorated chromosome is an iso (1p) as demonstrated 30 by DAPI-banding (open arrows). X 1200

Figure 7 shows CISS hybridization of chromosome 4 library inserts detected with a avidin-FITC; interphase nuclei of TC 593 (A-C) and TC 620 (D). Note that the two apparently complete interphase

05 domains are close to each other in B, but widely separated in A and C. TC 620 interphase nucleus (D) shows four chromosome 4 interphase domains of largely different sizes. Metaphase spread of TC 593 (E) shows two apparently complete 4 chromosomes, and

10 a small decorated region (arrow) in a submetacentric chromosome. This marker with translocated 4 sequences was observed in about 30% of the spreads. TC 620 metaphase spread (F) shows one apparently complete chromosome 4 and three translocation

15 markers (t) containing different amounts of chromosome 4 material. G-J Double hybridization of biotinylated chromosome 7 inserts and an aminoacetylfluorene (AAF)-modified 7-specific alphoid repeat. G Chromosome 7 inserts depict five

20 entirely decorated metaphase chromosomes. Four of them are complete 7 chromosomes, the fifth (arrow) is an iso (7p) (see Fig. 3E). H The same field as G showing AAF-7 alphoid signals on only four decorated chromosomes; no signal is detected on the iso (7p).

25 I An interphase nucleus of TC 593 shows five domains delineated by chromosome 7 inserts; four of these are labeled by 7 alphoid probes (J). The arrow in I represents the iso (7p) marker in interphase.

Figure 8 shows CISS hybridization of library

30 inserts of chromosomes 7 and 18 to metaphase spreads of glioma cell lines detected with avidin-FITC. X

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875. A, B TC 620 hybridized to chromosome 7 inserts. Three apparently normal 7 chromosomes and an additional translocation chromosome containing 7 sequences are indicated by large arrowhead in A;

05 DAPI-stained complete chromosomes are indicated by the small arrows in B. Other studies (see the text) indicated a translocation of 7pter-q11 in the marker chromosome (large arrowhead). D, The corresponding frame counterstained with DAPI. E, Metaphase spread

10 from pseudotetraploid TC 593 shows five chromosomes highlighted by 7 library inserts. The metacentric chromosome (m) represents the iso. (7p) marker typical for this line (see also Fig. 2G). Insert chromosomes (small arrows) show DAPI-stained normal

15 and metacentric 7 chromosomes. The landmark band 7q21 and a block of constitutive heterochromatin at 7q11 are both prominent on the normal chromosome 7 insert, (arrows) but not present on the marker chromosome. Instead both arms of the latter show a

20 mirror-like staining pattern with a faint distal band at 7p21. F, G TC 593 hybridized to chromosome 18 inserts. Four decorated 18 chromosomes are shown in F and three of them in G by DAPI staining are clearly translocated (t).

25 Figure 9 is a summary chromosome idiogram of complete and aberrant chromosomes detected by CISS hybridization of library inserts of chromosomes 1, 4, 7, 18 and 20 in glioma cell lines TC 620 (left) and TC 593 (right). G-bands (black) are shown with

30 approximate breakpoints suggested by our data; the shaded areas with a wavy pattern are from other

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chromosomes that constitute part of the marker translocation chromosomes. The black dot beside two of the TC 620 translocated 4 segments indicates that the assignment of the chromosome 4 material is based
05 on circumstantial evidence (e.g., size measurements). A small translocation of chromosome 18 material in ca. 20% of TC 593 metaphase spreads (+) also could not be further identified. Note the over-representation of 7p in both cell lines.
10 Figure 10 shows representative prophase (A) and interphase nuclei (B-F) reflect metaphase abnormalities in glioma lines (cf. Figs. 1-3). A, Detection of the 7p translocation (t) in a prophase TC 620 nucleus. X 1,000. B, Five well-separate
15 chromosome 7 domains detected in TC 593. X 1,240. C, Two large and one very small 18 domains (indicated by arrow head) in TC 620. X 1,450. D, Four chromosome 18 domains detected in TC 593; one of these signals (arrow) appears smaller. X 1,450.
20 E, Four chromosomal 1 domains in TC 620 (cf. Fig. 1A, B). X 1,200. F, At least five chromosome 1 domains in TC 593; one (arrow head) is appreciably smaller than the others. X 1,250. G, H, CISS hybridization of a technically poor metaphase spread
25 of TC 593 poststained with DAPI (G) still highlights four distinct chromosomes bearing 18 sequences (H). X 1,000.

Figure 11 is a graphic representation of the interphase and/or metaphase counts of chromosomes 7
30 (A-C), 22 (D) and 4 (E) by CISS hybridization. Interphase counts were performed on 150 nuclei of

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well-hybridized preparations. For metaphase counts > 25 complete DAPI-stained spreads were evaluated. A-C Counts of 7 specific alphoid repeats (white columns) compared to 7 library inserts (shaded columns): A, interphase nuclei of phytohemagglutinin-stimulated human lymphocytes (46,XY); B, TC 620 interphase nuclei (7-specific alphoid repeat) and metaphase spreads (7 library inserts); C, TC 593 interphase nuclei (7-specific alphoid repeat) and metaphase spreads (7 library inserts). High stringency hybridization (see Materials and methods) of 7 alphoid repeat was used to avoid cross-hybridization to other chromosomes. In cases of double hybridization with both 7 library inserts and alphoid repeat (shown in Fig. 2 G-I) standard conditions with 50% formamide were sufficient to avoid cross-hybridization, possibly due to the presence of human competitor DNA. D, Counts of chromosome 22 (library inserts) in metaphase spreads of TC 620 (black columns) and TC 593 (shaded columns). For comparison, CISS hybridization was simultaneously performed with 7 library inserts in these experiments as an internal control (see C,D and Fig. 7). E, Interphase counts (white columns) and metaphase counts (shaded columns) compared in TC 593 hybridized with chromosome 4 inserts. Note the ratio of signal preparations 2:3 are the same in metaphase and interphase.

30 Figure 12 shows a TC 620 metaphase spread after double hybridization with inserts from chromosomes 7.

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and 22 (both labeled with biotin and detected with avidin-FITC). Two strongly decorated 22 chromosomes (arrows), three complete 7 chromosomes, and the metacentric marker chromosome containing 7pter-q11
05 are also seen.

Figure 13 is a graphic representation of the relative size of decorated normal and aberrant chromosomes 4, 7 and 18 in typical metaphase spreads (n=24) from glioma cell lines TC 593 and TC 620.
10 Individual areas were normalized so that a complete chromosome is represented by an area of 1 (see legend to Table 1). The total added signals reflect the number of specific chromosome equivalents present. The white regions correspond to apparently
15 normal chromosomes, the black regions indicate small free chromosome segments entirely decorated by specific library inserts, and translocated segments are shaded. One of the three translocated 18 chromosomes in TC 593 represents a complete
20 chromosome by this measurement (indicated by the black dot), while the two other translocations are slightly smaller, possibly due to the small sample size.

Figure 14 shows specific labeling of human
25 chromosome 21 by CISS hybridization with biotinylated DNA probe sets. A, plasmid pPW519-1R (6-Kb insert) hybridized to a normal lymphocyte metaphase spread. Signals are located on the termini of 21q (see 4',6-diamidino-2-phenylindole
30 (DAPI)-stained chromosomes in Inset) as verified by DAPI banding (not shown). B and C, Normal human

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lymphocyte metaphase (B) and nuclei (C) after hybridization with the 94 Kb plasmid pool probe set. The terminal band 21q22.3 is specifically labeled. 05 D and E, Signals on trisomy 21 (47, + 21) lymphocyte metaphase spreads after hybridization with the 94 Kb probe set (D) or chromosome 21 library DNA inserts with the CISS hybridization (14) protocol. (E) Three chromosomes 21 are entirely delineated by the library inserts; additional minor signals (see text) 10 are indicated by arrowheads (also in G). (F-J) Labeling of trisomy 21 lymphocyte nuclei by the library inserts (F and G; compare with E).

Detailed Description of the Invention

The present invention is based on a hybridization strategy in which suppression of hybridization signals from ubiquitous repeated DNA sequences is achieved by using total DNA in a reannealing procedure which is based on rapid reassociation kinetics. 15 The hybridization method of the present invention referred to as chromosomal in situ suppression (CISS) hybridization because of the selective suppression of such signals, has been shown to result in specific cyto-staining of one or more selected individual chromosomes, particularly human 20 chromosomes, at any point in the cell cycle and has been used to detect, identify and quantitate chromosomal aberrations in both mitotic cells and interphase cells (i.e., interphase nuclei).

25 Described below and in greater detail in the Examples, are the following:

1. specific staining in mitotic and interphase cells of individual human chromosomes, by the method of the present invention (CISS hybridization), using chromosome-specific probe sets which are of high genetic complexity (i.e., chromosome library DNA, cloned DNA fragments);
05
2. specific staining of metaphase and interphase tumor cells by CISS, using chromosome-specific library probes; and
10
3. rapid detection in mitotic and interphase cells from a variety of sources of aberrations in a human chromosome (chromosome 21) which is associated with a genetic condition (Down syndrome), using CISS hybridization.
15
4. demonstration that a nested set of chromosome specific unique sequence probes has been used to identify chromosome aberrations and to detect genetic disease (e.g., Down Syndrome).
20

Specific Staining of Individual Human Chromosomes
By use of the CISS hybridization method, individual (such as the X and Y chromosomes or homolog pairs of chromosomes 1-22) human chromosomes have been specifically stained in both mitotic and 25 interphase cells. This has been carried out in both metaphase spreads and interphase nuclei and has been used to stain or label one selected (individual) chromosome and to stain or label multiple selected (individual) chromosomes simultaneously, using, 30 respectively, signal-probe CISS hybridization and

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multi-probe CISS hybridization in conjunction with an appropriate detection method. The method is represented schematically in Figure 1.

Specific Chromosome Staining Using Genomic DNA

05 Libraries and Cloned DNA

CISS hybridization was carried out as follows, to produce specific staining of individual human chromosomes, using commercially-available genomic DNA libraries that originated from flow-cytometry sorted human chromosomes and cloned DNA fragments.

10 Van Dilla, M.A. et al., Biotechnology, 4:537-552 (1986). Suppression of hybridization signals from ubiquitous repeated sequences, such as the Alu and KpnI elements, was achieved using total human DNA in 15 a reannealing procedure that is based on rapid reassociation kinetics. Similar principles have been used by others to facilitate the selective hybridization of unique sequence subsets from cosmid DNA clones for Southern blotting and in situ 20 hybridization experiments. Sealey, P.G. et al., Nucleic Acids, 13:1905-1922 (1985); and Landegent, J.E. et al., Hum. Genet., 77:366-370 (1987).

25 Specific labeling of individual chromosomes in both metaphase spreads and interphase nuclei, is carried out (and shown to have occurred) in the following manner, which is described in detail in the Examples. The feasibility of using computer-assisted optical sectioning for 3-D reconstruction of chromosomal domains for the

analysis of nuclear topography was also demonstrated in conjunction with CISS hybridization.

Initially, genomic DNA from a selected chromosome or selected chromosomes is prepared for use as probe DNA. Genomic DNA is available from several sources. For example, one or more genomic DNA libraries, each containing the chromosome of interest (a chromosome-derived library), is used to produce the necessary DNA probes. Such libraries can be commercially-available genomic DNA libraries that originated from flow-cytometry sorted human chromosomes. These are available from the American Type Culture Collection (Rockville, MD). Such DNA libraries for human chromosomes 1, 4, 7, 8, 9, 12, 13, 14, 16, 17, 18, 20, 21, 22 and chromosome X have been used in the present method, as described in the Examples. Other commercially available genomic DNA libraries or genomic DNA libraries from noncommercial sources can also be used.

Alternatively, individual plasmid, phage, yeast artificial chromosomes with non-yeast DNA inserts, and cosmid DNA clones can be used as a source of DNA probes for a selected individual chromosome or multiple selected chromosomes. In the case of DNA from a genomic library, the DNA can be separated as a pool from the vector containing it, prior to labeling with a detectable signal, or can be used without separation from the vector.

Probes are labeled with a detectable signal, which can be a fluorescent reporter, one member of a specific binding pair (e.g., biotin-avidin or

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ligand-antibody), or an enzyme. DNA removed from the vector is labeled by nick translation (using, for example, Bio-11-dUTP), by random primer extension with (e.g., 3' end tailing), for example, 05 the Amersham multiprime DNA labeling system, substituting dTTP with Bio-11-dUTP, or other appropriate technique. In the case of DNA which has not been separated from the vector, biotin labeling is carried out directly by nick translation, using 10 standard techniques. Brigati *et al.*, Virology, 126:32-50 (1983). Other labels can be added in a similar manner (e.g., 2,4-dinitro phenol, digoxin).

Probe size is carefully selected and controlled in order to facilitate probe penetration and to 15 optimize reannealing hybridization. In general, labeled DNA fragments smaller than 500 nucleotides are used, and, more generally, the majority of the probes are 150-250 nucleotides in length. Probes of this length are made from longer nucleotide 20 sequences using publicly available restriction enzymes or known techniques for producing and recovering appropriately-sized fragments. It is also possible, if the nucleotide sequence of a selected chromosome is known, to synthesize an 25 oligonucleotide having that sequence, using known techniques. Such oligonucleotides, once labeled, can be used to decorate specific chromosomal regions. For example, oligonucleotide probes which specifically hybridize to telomeric sequences of 30 mammalian chromosomes have been identified. Moyuif

et al., Proceedings of the National Academy of Sciences, USA, Sept. 1988.

Competitor DNA, which is DNA which acts to suppress hybridization signals from ubiquitous 05 repeated sequences, will be selected as needed (e.g., based on the mammal whose chromosomes are being analyzed). In the case of analysis of human chromosomes, competitor DNA is total human DNA which acts to suppress hybridization from ubiquitous 10 repeated sequences, such as the Alu and the KpnI elements. It is available from many sources. For example, human genomic DNA from placenta or white blood cells can be prepared using known techniques, such as that described by Davis et al. Davis, L.G. 15 et al., Basic methods in molecular biology, Elsevier, N.Y./ Amsterdam (1986). It is digested, using standard methods (e.g., with DNase), to produce competitor DNA fragments within the same size distribution as the probe DNA.

20 DNA from another source, which will compete with only a small portion of the human DNA and which is used, as necessary, to adjust the total (final) DNA concentration of the hybridization mixture will also be included, as needed. This DNA is referred 25 to as carrier DNA. This DNA is produced or treated, using standard methods, so that it is within the same size distribution as the probe DNA.

Initially, probe DNA bearing a detectable label and competitor DNA are combined under conditions 30 appropriate for preannealing to occur. The quantity of probe DNA combined with competitor DNA is

adjusted to reflect the relative DNA content of the chromosome target. For example, chromosome 1 contains approximately 5.3 times as much DNA as is present in chromosome 21. Probe concentrations were 05 30 μ g/ml and 5 μ g/ml, respectively. When total genomic library DNA is used as the probe mixture (instead of purified DNA inserts), approximately 10 times as much labeled DNA is added to compensate for the vector sequences, which are present in large 10 quantities. Only twice as much labeled library DNA is added in the case of the libraries LAOXNL01 (X chromosome) and LA16NL02 (chromosome 16) because the human DNA inserts constitute almost half of the total library DNA. Carrier DNA, such as trout or 15 salmon testis DNA, is added to bring the total DNA concentration to a predetermined level, if necessary. As described herein, sufficient salmon testis DNA was added to result in a final DNA concentration of 1.0 mg/ml in the hybridization mixture (which 20 includes all three types of DNA: probe DNA, competitor DNA and DNA which does not significantly compete).

The resulting hybridization mixture is treated (e.g., by heating) to denature the DNA present and 25 incubated at approximately 37°C for sufficient time to promote partial reannealing.

The sample containing chromosome DNA to be identified (specifically labeled) is also treated to render DNA present in it available for hybridization 30 with complementary sequences, such as by heating to denature the DNA. The hybridization mixture and the

sample are combined, under conditions and for sufficient time conducive to hybridization. After sufficient time, detection of specific labeling of the chromosome target is carried out, using standard

05 techniques. For example, as described in the Examples, a biotinylated probe is detected using fluorescein-labeled avidin or avidin-alkaline phosphatase complexes. For fluorochrome detection, samples are incubated, for example, with fluorescein 10 isothiocyanate (FITC)-conjugated avidin DCS (see Example 1). Amplification of the FITC signal can be effected, if necessary, by incubation with biotin-conjugated goat anti-avidin D antibodies, washing and a second incubation with FITC-conjugated avidin.

15 For detection by enzyme activity, samples are incubated, for example, with streptavidin, washed, incubated with biotin-conjugated alkaline phosphatase, washed again and pre-equilibrated (e.g., in AP-buffer, as described in Example 1). The enzyme

20 reaction is carried out in, for example, AP buffer containing nitroblue tetrazolium and 5' oromo 4 chloro 3 indoyl phosphate and stopped by incubation in 2 XSSC.

Detection of Chromosome Aberrations Using CISS

25 Hybridization

Using the above-described steps, it is possible to specifically stain or label any selected individual chromosome (or chromosomes) referred to as a target chromosome, or a subregion(s) thereof. As 30 explained in the examples, the present method has

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been shown to be useful in a variety of cells, both in mitotic (e.g., metaphase, prophase) and interphase cells. As described in detail in Example 2, the CISS hybridization method of the present method 05 is useful for rapidly screening mitotic and interphase aneuploid tumor cells for complex numerical and structural aberrations of individual chromosomes (e.g., changes in number of chromosomes, deletions and rearrangements or translocations).

10 In this context, biotinylated library DNA inserts were used in the CISS hybridization method to produce hybrid molecules which were detected using known techniques. Two glioma lines were used as general models of aneuploid cells, particularly 15 tumor cells. One was an oligodendrogloma line and the other was a glioblastoma line. These were analyzed, using the biotinylated DNA probes specific for chromosome 1, 4, 7, 18 and 22. Specific labeling of the chromosomes, from pter to qter, made 20 it possible to visualize numerical changes, deletions and rearrangements in these chromosomes in metaphase spreads and in early prophase and interphase nuclei. Complete chromosomes, deleted chromosomes and segments of translocated chromosomes 25 were rapidly delineated in the very complex karyotypes of such cells. Additional subregional probes were also used to further define aberrant chromosomes. Digital image analysis was used to quantitate the total complement of specific 30 chromosomal DNAs in individual metaphase and interphase cells of each line. Under-representation

of chromosome 21 and over-representation of chromosome 7 (specifically 7p) were observed. This is in agreement with previous observations by others using conventional cytogenetic banding techniques.

05 Bigner, S.H. et al., Cancer Genet. Cytogenet., 22:121-135 (1987); Shapiro, J.R., Semin. Oncol., 13:4-15 (1986).

The two glioma cell lines used display several cytogenetic features common to many glioma cells.

10 Thus, it is reasonable to expect that the CISS hybridization method can be used in a similar manner to specifically decorate other chromosomes and to detect those chromosomes in glial tumors. The two cell types analyzed are highly aneuploid (i.e., they
15 have 100 chromosomes, rather than the normal 46). Therefore, it is reasonable to expect that the CISS hybridization method can be used in assessing any type of aneuploid (tumor) cell.

Thus, the CISS hybridization method can be used
20 in assessing chromosomal aberrations associated with cancer, both in diagnosis of the disease and in monitoring its status (.e.g., progression, regression or change with treatment) in patients. In this application, assessment of a single chromosome or of
25 multiple chromosomes, and subregions thereof, can be carried out. Double hybridizations using two DNA probes, each bearing a different label can also be carried out. That is, biotinylated chromosome 7 library DNA inserts and a probe specific for alphoid
30 repeats on chromosome 7 (pa7t1) which was modified with aminoacetylfluorene (AAF) were used to assess

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chromosome 7 content/characteristics in both meta-phase spreads and interphase nuclei of the two types of tumor cells (TC 593, TC 620). After hybridization, biotinylated chromosome 7 inserts were 05 detected using avidin-FITC and chromosome 7-specific alphoid AAF labeled sequences were detected with tetramethylrhodamine isothiocyanate (TRITC) conjugated second antibodies. Double CISS hybridization was used to detect translation between chromosome 8 10 and 14, Burkitt lymphoma cells, a high malignancy form of B lymphocyte tumors such were seen in both metaphase spreads and interphase cells.

This made it possible to detect similarities and differences in chromosome number 7 present in 15 the two tumor cell types: only the four complete number 7 chromosomes found in TC 593 contained a detectable 7 centromeric signal; a smaller and metacentric number 7 chromosome lacked the 7 alphoid sequences and a small block of heterochromatin at 20 7q11 (indicating that it lacked a characteristic centromeric region). In contrast, all four chromosome number 7 of TC 620 were labeled with the 7 alphoid probe. Double CISS hybridization also made it possible to distinguish among number 7 25 chromosomes present in one cell type (TC 593) and to demonstrate similarity (at least as to the characteristics assessed) among number 7 chromosomes present in the other cell type (TC 620).

Double CISS hybridization was used to detect 30 translocations between chromosome 8 and chromosome 14 in Burkitt's lymphoma cells; Burkitt's lymphoma

is a highly malignant form of B lymphocyte tumors. Translocations were detected in both metaphase spreads and interphase cells.

It is possible, through the use of appropriately-selected probes and/or labels to increase the number of different chromosomes, as well as the number of subregions on some or all of those chromosomes, which can be analyzed simultaneously using multiple CISS hybridization. For example, it is possible to use more than one probe, each specific for a subregion of a target chromosome, to analyze several subregions on that single chromosome at one time. It is also possible to label each probe set (set of DNA or RNA fragments) with a distinct fluorochrome or different reporter molecule, which can be distinguished from one another, after probe-target chromosome hybridization has occurred, by known techniques (e.g., by using specific fluorescent or enzyme reagents).

Furthermore, a "combinatorial" variant of CISS hybridization can be used to enhance the number of chromosomes which can be assessed simultaneously. That is, it is possible to use a hybridization probe mixture made from a single set of probe sequences composed of two halves, each separately labeled with a different fluorochrome (e.g., fluorescein and rhodamine), which, upon hybridization, produce a third fluorescence "color" or signal optically distinguishable from each of the original individual fluorochromes. Pairing of two different fluorochromes in this manner makes it possible to

identify three different chromosomes. For example, a probe set labeled only with fluorescein will yield one color upon hybridization; the same probe set labeled only with rhodamine will yield a second

05 (different) color upon hybridization. When half of the probe set is labeled with one of the two, both sequence subsets can hybridize to target with equal probability and be perceived as a third (different) color (in a way not dissimilar to mixing paint). It

10 is important here that two fluorochromes are not introduced into the same molecule, in order to minimize the possibility of E transfer (a well-known process where light emitted by one fluorochrome whose spectrum overlaps that of the other

15 fluorochrome is absorbed by the second fluorochrome. The transferred electrons are emitted by second fluorochrome, which leads to quenching of the first fluorochrome. Pairwise combinations of three different fluorochromes selected for their spectral

20 characteristics can be used singly and in pairwise combinations to produce in a similar manner. This can result in the production of six different fluorescent colors or signals (e.g., three pairs plus three single fluorochromes). Similar

25 combinations of four different fluorochromes results in production of 10 different fluorescent colors or signals, of five different fluorochromes results in production of 15 different colors or signals, etc. This principle of combinatorial fluorescence

30 (combining two or more fluorochromes to label the same probe set) is applicable to metaphase and

interphase chromosome analysis because each chromosome is a physically separate entity and is, thus, a distinct target. Composite probe labeling in which mixtures of three different fluorochromes 05 are used provides even greater diversity of colors or signals useful in simultaneous multiparameter analysis.

Another approach to enhance the number of chromosomes which can be analyzed simultaneously 10 involves a "time-resolved" method of fluorescence detection. In this instance, the DNA (or RNA) probes are labeled with chelating "cages" which bind specific lanthanides (e.g., Europium, terbium). Such metal chelates can be made to fluoresce. They 15 exhibit excited state lifetimes that are much longer (micro to millisec) than those of most normal fluorochromes (whose half lives are in the nanosecond range). Both the wavelength and the fluorescence lifetime is influenced by the nature of 20 the lanthanide metal ion employed. If a pulsed-gating system, which excites the sample with light for a few nanoseconds and then shuts off is used, it is possible to let short-lived fluorochromes decay to their ground state, open the 25 detector system at a defined time after excitation, (i.e., 1-100 microseconds) and detect only long-lived fluorochrome. This method can be used to discriminate 2 fluorescent dyes which have identical spectra but different lifetimes, thus adding a time 30 factor to fluorochrome discrimination.

Another approach to increase the number of different chromosomes that can be analyzed simultaneously is based on a detection system which distinguishes chromosomes in terms of the flexibility or rigidity of an attached fluorochrome. 05 Here, two single stranded probe sets can be labeled with the same fluorochrome, in one probe set the fluorochrome is introduced into the body of DNA sequences which will form hybrid molecules with the 10 target DNA of interest. In the second probe set, the fluorochrome is introduced into DNA sequences, that do not hybridize with the target DNA (e.g., by adding a 3'-tail of poly dA-fluorochrome with deoxynucleotide terminal transferase, ligation of 15 fluorochrome-labeled heterologous DNA to the probe DNA or other conventional secondary labeling techniques known in the art). Fluorochromes within the body of the DNA which form probe-target chromosome hybrids will become immobilized and thus will be 20 unable to rotate freely in solution. In contrast, fluorochromes in the single-strand DNA that is not involved in hybrid formation are not immobilized and can rotate much more freely in solution. By measuring the rate of fluorochrome rotational freedom, 25 (i.e., by measuring how fast the fluorochromes become depolarized when illuminated with polarized light) one can discriminate the two sets of probes.

Use of CISS hybridization and regionally defined probe sets for rapid assessment of chromosome aberrations associated with genetic disorders and chromosomal damage

05 It has been demonstrated that the CISS hybridization method is useful for the rapid assessment of chromosome aberrations (such as numerical and structural aberrations of chromosome 21) associated with genetic disorders (e.g., in the case of chromosome 21, Down syndrome). DNA probe sets which specifically label the terminal band 21q22.3 or decorate the entire chromosome 21 aberrations in metaphase and interphase cells are described in Example 3. The cloned DNA fragments from the human 10 chromosome 21 are useful to specifically label the cognate chromosomal region in metaphase spreads and interphase nuclei in a variety of cell types. That is, CISS hybridization using a chromosome 21 probe 15 set was shown to be effective in labeling/identifying chromosome 21 DNA in lymphocytes, embryonic chorionic villi cells and a glioma tumor cell line (TC 620). Unique probe sets from band 21q22.3 were 20 also used to detect chromosome 21 in solid tissue ("normal" human brain tissue). Thus, CISS 25 hybridization and hybridization with pools of unique sequence probes clearly have potential as a diagnostic for Down syndrome and for other genetic diseases or other conditions associated with chromosomal aberrations.

30 Results demonstrate that a trisomic karyotype can be diagnosed easily in interphase cells because the majority of the nuclei (55-65%) exhibit three distinct foci of hybridization. In contrast, less

than 0.2% of nuclei in lymphocytes with a disomic karyotype show three nuclear signals; interestingly, the percentage of such nuclei in normal CV cells was higher but still considerably less than 5%. In 05 general, as few as 20-30 cells were sufficient to unambiguously distinguish between disomic and trisomic cell populations. However, in view of the uncertainty of the level of chromosome 21 mosaicism in clinical samples, the number of cells required to 10 make an unambiguous diagnosis will likely be higher. Additional clinical correlations will be required to establish the absolute number. Nevertheless, this analytical approach could allow the diagnosis of Down syndrome without the need to culture cells or 15 to obtain metaphase spreads. It would also decrease the time required to make the diagnosis, from the current 10-14 days to 1 day or less.

Although selected plasmid clones containing only unique human DNA sequences were used here, 20 cosmid clones containing repetitive sequences can also be used to specifically label their cognate genomic region in metaphase and interphase cells by applying hybridization protocols like CISS hybridization that suppress the signal contribution 25 of repetitive sequence elements. Therefore, single or nested sets of cosmids could be used as diagnostic tools for other genetic diseases in a fashion similar to that reported here. Trisomy of chromosomes 13, 18 and 21 and numerical changes in 30 chromosomes X and Y together account for the vast majority of numerical chromosome abnormalities

identified during prenatal karyotyping. With the continued development of multiple nonisotopic probe labeling and detection systems it should be possible to visualize three or more chromosomes

05 simultaneously following in situ hybridization. The variations, described in the previous section, of the CISS hybridization method which increase the number of chromosomes, and/or the number of chromosome regions which can be assessed

10 simultaneously can also be used for detecting chromosomal aberrations associated with genetic disorders and chromosomal damage. Thus, the development of a rapid and automated screening test to detect the major trisomic disorders directly in

15 interphase cells from amniotic fluid or chorionic villi cells is a viable future objective. The analysis of specific human chromosomes by in situ hybridization has already been used to complement conventional cytogenetic studies of highly aneuploid

20 tumor lines (Example 2) and the extension to prenatal diagnostic applications seem warranted.

The analysis of karyotypes with translocations of chromosome 21 shows the usefulness of a regional probe set to rapidly identify and characterize even

25 small translocations by unambiguous signals on metaphase chromosomes, thus circumventing an extensive analysis by high-resolution banding. In contrast, the library insert probe is more suitable for defining the relative amount of chromosome 21

30 DNA that has been translocated. By analyzing interphase nuclei, one can also determine if a

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balanced or unbalanced number of chromosomal regions exists. However, the detection of a translocated chromosome directly in nuclei would require double-labeling techniques to identify the recipient
05 chromosome to which the chromosome 21 material was translocated. With prior knowledge of the chromosome in question, such translocation events could be assessed by measuring the juxtaposition of the nuclear signals. Rappold, G.A. et al., Hum. Genet.,
10 67:317-325 (1984).

A cosmid clone spanning the entire muscular dystrophy (MD) locus on chromosome X has been used to identify translocation between chromosome X and chromosome 4.

15 Probes containing 6 kb of sequence were localized in both metaphase spreads and interphase cells with high efficiency. This detection sensitivity with nonisotopic reagents is similar to that achieved in other recent reports. The combination
20 of nonisotopic in situ hybridization with DAPI or BrdUrd banding or total chromosome decoration with library DNA probes thus provides a simple and general approach for gene mapping. Combinatorial fluorescent technology will also make it possible to
25 examine several chromosomal regions simultaneously, thus permitting genetic linkage analysis by in situ hybridization. It also should facilitate the use of small DNA probes to rapidly pinpoint the breakpoints on translocation chromosomes, which could further
30 aid in defining the genomic segments critical for Down syndrome.

Identifying and Isolating Chromosome-Specific Sequences Using CISS Hybridization

The CISS hybridization method of the present invention can also be used to identify

05 chromosome-specific sequences and, subsequently, to separate them from repetitive sequences, using known techniques. Such chromosome-specific sequences, separate from the non-specific or repetitive sequences, and labeled, can be used in hybridization

10 assays carried out, for example, in a diagnostic context, to identify, detect, and/or quantitate a chromosome or chromosome region of interest (e.g., one which is associated with a genetic disorder or causes an infectious disease). Combination of a

15 sample to be assayed for a selected target nucleic acid sequence or sequences and appropriately-selected, labeled chromosome-specific sequences separated from repetitive sequences (e.g., sequences specific for sequences on the chromosome(s),

20 generally referred to as target nucleic acid sequences, which are to be detected and/or quantitated in the sample under appropriate conditions results in hybridization with complementary sequences present in the sample.

25 Hybridization will not occur, of course, if complementary sequences are not present in the sample.

Such separated chromosome-specific nucleic acid sequences can be incorporated into a kit to be used

30 for identification, detection and/or quantitation of chromosomes or chromosome regions of interest, using

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standard hybridization techniques. For example, labeled nucleic acid sequences which are chromosome 21 specific (or specific to a portion of chromosome 21), identified by CISS hybridization, and separated 05 from repetitive sequences present on chromosome 21, can be included in a kit, along with other reagents such as buffers, competitor DNA, carrier DNA and substances needed for detection of labeled chromosome 21-derived nucleic acid sequences 10 hybridized to chromosome 21 sequences present in a sample. Such kits clearly can be produced to include chromosome-derived nucleic acid sequences from one or more chromosome(s) of interest. Competitor DNA, carrier DNA and substances useful 15 for detecting hybridized sequences will be as described above.

EXAMPLE 1 Cyto-Specific Staining of Individual Human Chromosomes Using Genomic DNA Libraries in CISS Hybridization

20 DNA libraries

The following human chromosome genomic libraries were obtained from the American Type Culture Collection: LA01NS01 (chromosome 1), LL04NS01 (chromosome 4), LA07Ns01 (chromosome 7), 25 LL08NS02 (chromosome 8), LA13NS03 (chromosome 13), LL14NS01 (chromosome 14), LL19NS01 (chromosome 18), LL20NS01 (chromosome 20), LL21NS02 (chromosome 21), LA22NS03 (chromosome 22), LA0XNL01 (chromosome X). Amplification of these phage libraries on agar 30 plates (using LE 392 cells as the bacterial host),

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purification of the λ phages and extraction of phage-DNA pools were carried out according to standard protocols. Maniatis, T. et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor 05 Laborabory, Cold Spring Harbor, NJ (1982).

Preparation of metaphase spreads and fibroblast cells

Phytohemagglutinin-stimulated lymphocytes from a normal adult male (46, XY) were cultured in 10 McCoy's 5A medium (GIBCO), arrested with Colcemid, treated with a hypotonic solution of 0.075 M KCl, fixed in acetic acid-methanol and metaphase spreads made by standard procedures. Low-passage normal human foreskin fibroblasts (46, XY) were grown on 15 microscope slides, fixed with paraformaldehyde, and permeabilized as described for study of preparations with a more intact three-dimensional structure. Manuelidis, L, Ann. NY Acad. Sci., 450:205-221 (1985).

20 Preparation of DNAs for in situ hybridization

Insert DNA probes. Genomic DNA fragments from the chromosomal DNA libraries were separated as a pool from the Charon 21A vector arms by digestion with the appropriate restriction enzyme [EcoRI (LA 25 libraries) or Hind III (LL libraries)], followed by preparative electrophoresis in 0.6% agarose gel. The insert fragments were isolated from gel slices by electroelution into an Elutrap (Schleicher and

Schuell) and further purified by Elutip-d column chromatography (Schleicher and Schuell). The DNA was then extracted with phenol/chloroform (1:1) and ethanol precipitated. This pool of DNA fragments 05 was labeled either by nick translation using Bio-11-dUTP or by random primer extension with the multiprime DNA labeling system (Amersham) substituting dTTP with 0.5 mM Bio-11-dUTP. Langer, P.R. et al., Proc. Natl. Acad. Sci. USA, 78:6633-10 6637 (1981) and Brigati, D.J. et al., Virology, 126:32-50 (1983). Alternatively, the DNA of the chromosome-specific libraries was biotin-labeled directly (without separation of the vector arms) by nick translation.

15 Probe size. To facilitate probe penetration and to optimize reannealing hybridization, it is desirable to have labeled DNA fragments smaller than 500 nucleotides; the majority of the probes are generally 150 to 250 nucleotides in length. DNase 20 concentrations were empirically established in nick-translation reactions to yield fragments in the desired size range and this was verified by agarose gel electrophoresis. Random primer extensions were also carried out under conditions which yielded a 25 comparable DNA size distribution.

Competitor DNA. Human genomic DNA (from placenta or white blood cells), prepared as described, as well salmon testis genomic DNA (Sigma) were digested with DNase to obtain fragments with 30 the same size distribution as the probe DNA, then extracted with phenol/chloroform and ethanol

precipitated. Davis, L.G. et al., "Basic methods in molecular biology", Elsevier, New York Amsterdam (1986). These competitor DNAs were used in varying ratios with probe sequences, as described with

05 reference to Figure 2.

Preannealing and hybridization. Under standard conditions, from 5 μ g/ml to 30 μ g/ml of biotin-labeled DNA, representing library insert fragments, and varying amounts of competitor DNAs

10 were combined, ethanol-precipitated and resuspended in formamide. The probe concentration was adjusted to reflect the relative DNA content of each chromosome target. For example, chromosome 1 contains approximately 5.3 times as much DNA as

15 chromosome 21; thus the probe concentrations used were 30 μ g/ml and 5 μ g/ml, respectively.

Mendelsohn, M.L. et al., Science, 179:1126-1129 (1973). When total library DNA was used as the probe mixture instead of purified DNA inserts,

20 10 times as much labeled DNA was added to compensate for the large amount of vector sequences. In the case of the X-chromosome library, LA0XNL01, only twice as much labeled library DNA was used, since the human DNA inserts constitute almost half of the

25 total DNA. For comparative purposes, the concentration of human competitor DNA in the hybridization mixture was varied from 1 to 1.0 mg/ml and salmon testis DNA was added as required to result in a final DNA concentration of 1.0 mg/ml in

30 50% formamide, 1 x SSC (0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0) and 10% dextran

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sulfate. These solutions were heated at 75°C for 5 min. to denature the DNA and then incubated at 37°C for various times to promote partial reannealing. The preannealing step was done in an Eppendorf tube 05 just prior to applying the hybridization mixture to the specimen. Nuclei and chromosome spreads on glass slides were incubated in 70% formamide, 2 X SSC] at 70°C for 2 min. to denature chromosomal DNA and then dehydrated in a series of ice-cold 10 ethanal (70%, 90% and 100%, each for 3 min.). After application of the preannealed probe mixture (2.5 $\mu\text{l}/\text{cm}^2$) to slides prewarmed to 42°C, a coverslip was added and sealed with rubber cement. The samples were then immediately incubated at 37°C in a moist 15 chamber for 10-20 h.

In those cases where paraformaldehyde fixation was used to more optimally preserve the 3-D structure of the specimen, the slides were equilibrated in 50% formamide, 1 X SSC (2 X 5 min.), excess fluid 20 was removed without permitting the sample to dry, the probe mixture was added (5 $\mu\text{m}/\text{cm}^2$), and a coverslip mounted and sealed with rubber cement.

Manuelidis, L., Ann. NY Acad. Sci., 450:205-221 (1985). Denaturation of both probe and cellular DNA 25 was done at 75°C for 5 min. before hybridization was allowed to proceed overnight at 37°C.

Detection

After hybridization, the slides were washed in 50% formamide, 2 X SSC (3 X 5 min., 42°C) followed 30 by washes in 0.1 X SSC (3 X 5 min., 60°C).

Thereafter the slides were incubated with 3% bovine serum albumin (BSA), 4 x SSC for approximately 30 minutes at 37°C. Detection of the biotinylated probe was achieved using either fluorescein-labeled 05 avidin or avidin-alkaline phosphatase complexes. All detection reagents were made up in 4 X SSC, 0.1% Tween 20, 1% BSA and all washes were carried out in 4 X SSC, 0.1% Tween 20 (3 X 3 min., 42°C). For fluorochrome detection, slides were incubated with 5 10 µg/ml fluorescein isothiocyanate (FITC)-conjugated avidin DCS (Vector Laboratories) at 37°C for 30 min., followed by washes. In rare cases, the FITC signal was amplified by incubation with 5 µg/ml biotin-conjugated goat anti-avidin D antibodies 15 (Vector Laboratories) at 37°C for 30 min., followed by washing, a second incubation with 5 µg/ml FITC-conjugated avidin (37°C, 30 min.) and a final wash. Pinkel, D. et al., Proc. Natl. Acad. Sci. USA, 83:2934-2938 (1986). For detection by enzyme 20 activity, samples were incubated with 2.5 µg/ml streptavidin, washed, incubated with 2 µg/ml biotin-conjugated alkaline phosphatase (Vector Laboratories), washed again and pre-equilibrated in Ap-buffer 9.5 (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 25 50 mM MgCl₂) for 2 x 5 min. at room temperature. The enzyme reaction was carried out in AP buffer 9.5 containing 330 µg/ml of nitroblue tetrazolium (NBT) and 165 µg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) at 37°C for 0.5-1 hour and stopped by incubation 30 in 2 X SSC. All preparations were counter-stained with 200 ng/ml 4,6-diamidino-2-phenylindole-

dihydrochloride (DAPI), 2 X SSC for 5 min. at room temperature and mounted in 20 mM Tris-HCl, pH 8.0, 90% glycerol containing 2.3% of the DAPCO antifade, 1,4 diazabicyclo-2(2,2,2)octane. Johnson G.D. et
05 al., J. Immunol. Methods, 55:231-242 (1982).

Densitometry

A graphics workstation (VAX station II/GPX, Digital Equipment Corporation) with a frame grabber (ITEX FG-101, Imaging Technology) and a Dage-MTI-65 10 video camera with a Zeiss S-Planar 60 mm lens were used as described in Manuelidis, L. and J. Borden, Chromosoma, 96:397-410 (1988). Images were digitized directly from the negatives and stored on disk. Background was removed and polygonal regions 15 around each chromosome were defined. Threshold density levels were used to outline chromosome regions within the defined polygonal areas. Means density levels within these outlined chromosome regions, R , were determined by the total signal 20 $\int I(x,y)dR/\text{area } R$, where $I(x,y)$ is the pixel intensity (0-225) at each point within the region R . The threshold background intensity was substrated from the mean regional density, both for labeled chromosome 7 and for background chromosomes. The 25 signal to noise ratio was calculated as mean chromosome 7 signal/mean background chromosome signal.

The following is a description of the results of the work described above, which clearly demonstrate specific labeling of the individual 30 chromosome indicated. The first sections describe

use of chromosome library inserts labeled with biotin and the second describes use of DNA insert fragments.

Figure 2 shows suppression of signals from 05 cross-reacting sequences within a chromosome 7-derived DNA library by different concentrations, as described below.

Figure 2A shows chromosome 7 library inserts labeled with biotin and hybridized to metaphase 10 spreads from normal human lymphocytes without human competitor DNA. Prominent labeling of the two no. 7 chromosomes is observed; additionally, a distinct band-like patterns of hybridization is seen on most of the other chromosomes, and two E-group chromo- 15 somes are especially brightly stained. This general chromosomal banding pattern resembles R-banding, and suggests that a significant portion of the back- ground cross-hybridization signal originates from Alu repetitive sequences. Previous studies have 20 shown that Alu sequences delineate an R-banding pattern, while Giemsa positive-banding profiles are highlighted by KpnI interspersed repeats.

Manuelidis, L. and C.D. Ward, Chromosoma, 91:28-38 (1984).

25 Establishment of experimental procedure to eliminate the hybridization signal from repetitive elements

A series of pilot studies were therefore undertaken to establish experimental parameters to eliminate the hybridization signal from such repetitive elements. The kinetics of nucleic acid 30

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reassociation in solution are dependent on the total concentration of nucleic acid (C_0 , in moles of nucleotides per liter) and the time of renaturation (t , in seconds). When reassociation conditions are 05 standardized for temperature (taking into account the formamide concentration), cation concentration and buffer system, the reassociation kinetics are comparable with respect to Cot values. Under defined conditions, the fast reassociating fraction 10 of mammalian genomes containing the highly repetitive DNA is completely reannealed at Cot values between 1×10^{-1} and 5×10^{-1} ; the intermediate fraction containing the middle repetitive DNA is completely renatured at a Cot value of 1×10^2 . 15 Britten, R.J. and D.E. Kohne, Science, 161:529-540 (1968). Thus at a human DNA concentration of 1.0 mg/ml (corresponding to 3×10^{-3} moles of nucleotide per liter), the fast fraction would be renatured in approximately 10s, whereas the middle repetitive DNA 20 would need more than 9 h to reach complete reannealing. Since the fast fraction of reassociating DNA containing most or all of the ubiquitous repetitive DNA causing cross-hybridization signals, a total DNA concentration of 1.0 mg/ml was used and 25 partial reannealing of the probe mixture was allowed prior to application to specimens. The optimal renaturation time was determined empirically (see below). This was important because the *in situ* hybridization conditions deviate from the standard 30 conditions under which reassociation kinetics are determined (e.g., hybridization in 50% formamide at

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37°C corresponds to 0% formamide at about 70°C; dextran sulfate also increases the reassociation time significantly). Furthermore, it was unclear to what degree the middle repetitive DNA contributed to the non-specific signal and therefore should also be prevented from hybridization by a preannealing procedure.

The stringency for the reannealing and *in situ* hybridization experiments was determined in 50% formamide at 37°C (adapted from standard *in situ* hybridization protocols) and 1 X SSC [this cation concentration of 0.165 M comes close to the concentration used in kinetics the study of Britten and Kohne. Britten, R.J. and D.E. Kohne, Science, 161:529-540 (1968)]. Competitor human DNA was added in the reassociation procedure to obtain the desired final high DNA concentration and to maintain a high level of repetition of the DNA sequences that should preanneal. While total human genomic DNA represents all the highly repetitive DNA to be removed by preannealing, it also contains sequences of the target chromosome. Thus, the addition of excessive amounts of human DNA would be expected to diminish the chromosome-specific signal. Therefore, the optimal concentration of total human DNA to use as the competitor was first determined. To keep the total DNA concentration constant at 1.0 mg/ml, genomic salmon DNA was added as needed. Salmon DNA shares certain repetitive DNA elements, such as poly dCdA in common with human DNA, but lacks others, most notably the Alu- and KpnI repeats. Hamada, H. et

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al., Proc. Natl. Acad. Sci. USA, 79:6465-6469 (1982). This results in a lower frequency of the latter sequences with increasing amounts of salmon DNA in the reassociation reaction.

05 Figure 2 shows typical experimental results obtained when 20 μ g/ml of the chromosome 7 probe set was denatured together with 50 μ g/ml (B), 100 μ g/ml (C), 200 μ g/ml (D) or 1000 μ g/ml (E) of DNase-digested human genomic DNA which was preannealed for
10 20 min. Hybridization and detection using avidin-FITC were carried out as described above. From each preparation ten black and white pictures were taken under standardized photographic conditions for densitometric studies (see below). In the absence
15 of human genomic competitor (A) the signal showed little chromosomal specificity. However, with 50 and 100 μ g/ml of human competitor DNA, as increase of label specificity is readily apparent (Figure 2B,C). Specific staining of chromosome 7 was
20 achieved with a peak of signal intensity using 100 and 200 μ g/ml of human competitor DNA (Figure 2C, D). Higher concentrations of human DNA caused an apparent decrease of signal intensity, especially at 1000 μ g/ml human DNA (Figures 2E). However, the
25 signal obtained under these latter conditions is still reasonably bright to the observer, but requires a different exposure for optimal illustration (not shown).

30 A computer-assisted method of quantitative densitometry (see above) was used to establish the overall level of labeling specificity. The ratio of

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fluorescence signal from the target chromosomes of
interest to the background fluorescence noise
emanating from non-target chromosomes was determined
from images digitized from multiple photographic
05 negatives of a DNA titration experiment, as illus-
trated in Figure 2. The signal-to-noise ratio
obtained with each concentration of human competitor
DNA is given in Table 1.

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Table 1 Densitometric analysis of the suppression of cross hybridization signals by concentrations of human competitor DNA

05	DNA conc. μg/ml)	Signal-to-noise ratio					
		Human competitor					
		DNA conc.	Signal Pixel ^a	Noise Pixel ^b	η	η	
10	0		71.48	8	54.66	26	1.31±0.04
	50		74.50	8	37.43	28	1.99±0.07
	100		162.64	8	20.06	23	8.11±0.35
	200		147.35	8	20.53	26	7.18±0.37
	500		89.78	8	18.63	21	4.82±0.28
15	1000		94.37	8	30.51	17	3.09±0.12

^a Mean value of pixel intensity of target chromosome

^b Mean value of pixel intensity of non-target

chromosomes (from the same metaphase spreads)

^c The confidence interval was calculated using

20 Fieller's theorem (Finney, D.J., Statistical methods in biological assay, 2nd edn., Hafner Press, N.Y., 1971)

η Number of chromosomes from which the mean was determined

25 Optimal reannealing conditions for suppression of nonspecific signal (using 20 μg/ml of chromosome 7 probe and 100-200 μg/ml human genomic (DNA), gave a

signal-to-noise ratio of ca. 8:1. Additional attempts to improve the signal to noise ratio by increasing hybridization stringencies (e.g., 60% formamide or 0.2 x SSC) gave no apparent improvement 05 and led to an overall decrease in signal intensity.

Since about 100-200 μ g/ml of human competitor DNA was shown to give the optimal specificity, 200 μ g/ml was used for another analysis of signal specificity with respect to the renaturation time 10 (see above). After 0, 2, 5, 10, 20, 40 and 50 min. of preannealing, aliquots were taken and used for in situ hybridization experiments as before. As indicated in Figure 3, specific labeling was obtained for all preannealing times. A small improvement 15 of the signal is seen with increasing renaturation times from 0 to 20 min. Longer renaturation times up to 60 min. (not shown) gave no significant improvement in signal strength or chromosome specificity. The subjective impression of a signal 20 improvement with 20 min. of preannealing (Figure 3D) could not be confirmed by a densitometric analysis, carried out as described above, since no significant differences in the signal-to-noise ratio of the different preannealing times were observed (data not 25 shown). Therefore, the standard renaturation time in all subsequent experiments was 10-20 min. Since a signal is clearly visible at renaturation time 0, the few seconds necessary for transferring the probe mixture to the microscope slide appear to be sufficient 30 to effectively preanneal many of the sequences that cause nonspecific labeling by

cross-hybridization. Furthermore, the large excess of single-stranded competitor DNA may efficiently compete with the biotinylated probe sequences for ubiquitous chromosomal target sites during the

05 hybridization reactions. These results demonstrate that the majority of highly repetitive DNA sequences can be sufficiently suppressed to achieve chromosome-specific labeling by *in situ* hybridization.

10 In certain cases the signal distribution over the entire chromosome shows some variability from experiment to experiment. When the overall signal is decreased, some chromosomal subregions show a brighter staining; these signal hotspots generally

15 constitute chromosomal sites that contain known chromosome-specific repetitive sequences. In the experiments shown in Figures 2 and 3, predominant staining of the centrometric region of chromosome 7 is seen, which corresponds to the chromosome-

20 specific signal of an alphoid repetitive DNA. Waye, J.S. et al., Mol. Cell Biol., 7:349-356 (1987) and see Example 2. Apparently, the abundance of these repeated sequences is sufficiently low to prevent their suppression under the conditions used here.

25 The unequal signal distribution can be overcome by amplifying the overall signal using an antibody sandwich technique as described above. Furthermore, a predominant staining of the region 1q12 that corresponds to the chromosomal site of satellite III

30 DNA was frequently observed in labeling chromosome 1. Cooke, H.J. and J. Hindley, Nucleic Acids Res.,

6:3177-3197 (1979) and Gosden, J.R. et al., Cytogenet. Cell Genet., 29:32-39 (1981) and see Example 2. An example of the balanced signal distribution seen after such an amplification step is shown in

05 Figure 4A.

Several commercially available DNA libraries, each representing a single human chromosome, were tested for their ability to specifically label the chromosome they represented, under the standardized 10 reannealing conditions described above and with the probe concentrations adjusted for chromosome size, as described above. Some examples, for chromosomes 1, 4, 7, 13, 18 and 20, as shown in Figure 4, clearly demonstrate that specific labeling can be 15 achieved with most chromosome libraries. Table 2 lists the libraries tested with their relative scores of labeling specificity. All scores are positive because the chromosome of interest was always decorated. The highest score (4+) is used 20 when no significant cross-hybridization to other chromosomes was observed and the scores decrease (3+ to 1+) with an increasing amount of cross-hybridizing sequences.

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Table 2 Relative quality of specific chromosome labeling in situ using preannealed biotinylated library DNA inserts

Chromosome	Library used (ATCC designation)	Relative specificity of in situ hybridization signal ¹
05	1 LA01NS01	3+
	4 LL04NS01	4+
	7 LA07NS01	4+
10	8 LL08NS02	4+
	13 LA13NS03	1+
	14 LL14NS01	2+
	18 LL18NS01	4+
	20 LL20NS01	4+
15	21 LL21NS02	3+
	22 LA22NS03	3+ ^b
	X LA0XNL01	4+

^a See the text for score definition

^b Under standard preannealing conditions the 20 chromosome 22 library gave a score of +1; a value of +3 was achieved only with a human competitor DNA concentration $\geq 700 \mu\text{g/ml}$ (total DNA concentration 1.0 mg/ml).

25 All attempts to reduce the additional signals on other chromosomes by varying the experimental conditions failed except in experiments with chromosome 22; in this case higher concentrations of human competitor DNA (700 $\mu\text{g/ml}$) resulted in a significant

improvement of signal specificity. The library exhibiting the lowest chromosome specificity was the chromosome 13 library (Figure 4E). Multiple minor binding sites on other chromosomes, as well as an

05 exceptionally bright staining of Yql2 were observed; the signal on the Y chromosome was visible using either female or male human DNA as the competitor. None of the experimental parameters tested improved on the overall specificity of this library.

10 Remarkably, a weak signal or even absence of signal can be observed at the centromeric region of some chromosomes (see chromosomes 4 and 18, Figure 4C,D). In contrast to chromosomes 1 and 7, which contain chromosome-specific repetitive elements, the

15 centromere regions of chromosomes 4 and 18 apparently contain repetitive sequences, most likely alphoid satellite DNAs, which are very abundant and thus are suppressed by the reannealing technique. However, these chromosomal regions are very small

20 and the effect can only be observed when the corresponding chromosomes are fairly elongated.

Biotinylated total library DNA (containing the phage vector sequences) was also used as probes, in concentrations adjusted to the amount of human DNA

25 inserts. (see above). One example is shown in Figure 4F with the chromosome 20 library. Although good staining of the chromosome of interest generally was achieved, significant nonspecific background on the entire slide was a common problem.

30 Similar results were obtained with plasmid libraries containing human DNA subcloned from the lambda phage

libraries. In contrast, there was no background problem with the total chromosome library LA0XNL01, which contains a significantly smaller proportion of vector sequences in the probe mixture since the size 05 of the human DNA inserts is much larger.

The suppression of repetitive sequences by this reannealing technique also permits the use of flow-sorted chromosome libraries to detect chromosomal domains within interphase nuclei. Typical 10 examples of results obtained after hybridization of chromosome 1, chromosome 7 and chromosome 18 probe sets to normal human lymphocytes after acetic acid-methanol fixation are shown in Figure 5. Discrete focal domains of hybridization signal are 15 seen with all libraries that had scores of 2+ or more (see Table 2).

Most nuclei ($n \geq 100$ per estimate) exhibited two domains (60%-70%); however, a significant number showed only a single domain (20%-30%) or no 20 hybridization signal at all (5%-10%). Accordingly, ca. 95% of male nuclei exhibited one and ca. 5% showed no hybridization signal when the X chromosome library DNA was used as probe. Notably, no nuclei with three domains were found with any of the 25 chromosomal probe sets tested. In contrast, all metaphase spreads showed the decoration of both chromosome homologs without exception. This interphase variability may reflect, in part, the close juxtaposition of two individual domains in 30 some cells, or the inability to resolve domains that actually occupy different areas within the nuclear

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volume but are unresolved when examined by two-dimensional imaging methods (see Fig. 5D; for discussion see also Cremer *et al.*, Exp. Cell Res., 176:199-220 (1988). The small number of nuclei

05 exhibiting no hybridization signal may be a reflection of suboptimal hybridization conditions. It is of interest to note that the size of the intranuclear domains correlates reasonably well with the relative size of the cognate metaphase

10 chromosome. These observations provide a definitive proof that the DNA of individual chromosomes exhibits a clear territorial organization in the interphase nucleus of a normal human cell.

15 Acetic acid-methanol fixed nuclear spreads, such as those shown in Fig. 5, clearly retain the territorial organization for each of the chromosomes examined; however, the nuclear structure is not optimally preserved. Additional studies with specimens that possess better preservation of 3-D

20 structure using paraformaldehyde fixed human diploid fibroblasts and a laser-scanning confocal fluorescence microscope assembly for 3-D image reconstruction have been done. The cells were fixed and permeabilized as described by Manuelidis and

25 hybridized with chromosome library probes as outlined above. Manuelidis, L., Ann. NY Acad. Sci., 450:205-221 (1985). The probe-competitor DNA mixture was applied directly to the slide and denatured at the same time as the cellular DNA.

30 Results showed the arrangement of the chromosome 7 domains in the nucleus and the frequently observed

helical structure of labeled chromatic within chromosome domains. The degree to which this helicity reflects true domain substructure or is an artifact reflecting preparation and fixation

05 procedures is currently being investigated. Nevertheless, this preliminary observations establishes the feasibility of using chromosome specific probes to analyze the topography of chromosomal domains in the interphase cells.

10 EXAMPLE 2 Detection of Chromosome Aberrations in Tumor Cells by CISS Hybridization Using Chromosome-Specific Library Probes Cells

15 TC 593 is a pseudotetraploid cell line (modal chromosome number, 83) established from a human glioblastoma; it grows in a flat, spreading fashion and contains many process. TC 620 is pseudotriploid with a modal chromosome number of 64 and was established from a human oligodendrogloma; it grows in

20 an epithelial fashion. Both cell lines have been described in detail. Manuelidis, L. and E.E. Manuelidis, In: Progress in Neuropathology, Vol. 4, 235-266, Raven Press, N.Y. (1979). The present experiments made use of subclones C2B (TC 593) and

25 C2B (TC 620) at approximately 180 passages after repeated subcloning from a single cell of the original tumor line cultured as previously described by Manuelidis and Manuelidis (see reference above). Standard hypotonic treatment and acid/methanol

fixation of the cells were employed. Cremer *et al.*, *Exp. Cell. Res.*, 176:199-220 (1988).

DNA Probes and Libraries

Phage DNA libraries from sorted human
05 chromosomes were obtained from the American Type
Culture Collection: LA01NS01 (chromosome 1),
LL04NS01 (chromosome 4), LA07NS01 (chromosome 7),
LL18NS01 (chromosome 18) and LA22NS03 (chromosome
22). Amplification of these libraries, isolation of
10 human DNA inserts and biotinylation were carried out
as described in Example 1. A probe specific for
alploid repeats on chromosome 7 (pa7tl) was the gift
of H. Willard and specifically decorates
pericentromeric heterochromatin of chromosome 7
15 under high stringency conditions (60% formamide).
Waye *et al.*, *Mol. Cell. Biol.*, 7:349-356 (1987);
Cremer *et al.* *Exp. Cell. Res.*, 176:199-220 (1988).
Some DNA probes were modified with aminoacetyl-
fluorene (AAF); and detected as described by Cremer
20 *et al.* for double labeling experiments. Landegent
et al., *Exp. Cell Res.*, 153:61-72 (1984); Cremer, R.
et al., *Exp. Cell Res.*, 176:199-220 (1988).

In Situ Hybridization and Detection of Hybridized
Probes

25 CISS hybridization with biotinylated library
DNA inserts and detection of hybrid molecules was
generally carried out using standard conditions, as
described in detail in Example 1. In double CISS
hybridizations using biotinylated chromosome 7

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library DNA inserts and the AAF-modified 7 alploid probe, the latter probe was heat denatured separately and only added to the hybridization mixture at the end of the reannealing step at a final 05 concentration of 10 μ g/ml (see Example 1).

Digital Image Analysis of Specifically Decorated Metaphase and Interphase Chromosome

A VAX station II/GPX graphics workstation (Digital Equipment Corporation) with an ITEX FG 10 100-Q frame grabber (Imaging Technology) were used as previously described together with a Zeiss S-Planar 60 mm lens and a Dage-MTI 65 video camera. Manuelidis, L. and J. Borden, Chromosoma, 96:397-410 (1988). Images were digitized from negatives of 15 metaphase spreads and interphase nuclei; the background was removed and polygonal regions were defined to specifically decorated metaphase chromosomes or interphase domains (see Example 1). A scan line algorithm was used to calculate histograms 20 within the polygonal regions. Since the value of the histograms $H(i)$ of a particular intensity (range 0-255) within the defined regions is the number of pixels at that intensity i , the area within the region falling within an intensity range $i_0 - i_1$ is 25 the integral of the histogram from $i_0 - i_1$. Similarly, the 2-D integral in the region defined by the intensity range $i_0 - i_1$ equals $\Sigma H(i).i.i_0$ was chosen for each hybridization, in order to properly outline the decorated chromatin and distinguish this 30 area from background regions. i_1 was set to the

maximum value 255 in order to capture the entire intensity range above the threshold.

Measurements of total signal intensity versus area were designed as a control for the potential presence of variable chromosome domain extension within interphase nuclei. In interphase, a more extended chromosome domain might be expected to have a greater area (or volume) yet a lower fluorescence signal intensity per unit area. If a constant amount of hybridized DNA corresponds to a constant total fluorescence, the total signal intensity is a measure of labeled DNA content. It is also possible to measure 3-D hybridized volumes within nuclei and 3-D integrated total hybridized signals.

Manuelidis, L. and J. Borden, Chromosoma, 96:397-410 (1988). The background, b , was subtracted from the discrete 2-D integral $\iint I(x,y)dA$ within a labeled region R , to yield the total signal:

$$\text{Sig}_t = \iint I(x,y)dA - b \iint dA, \text{ where } dA \text{ is a single pixel.}$$

Similarly, the mean intensity within the region is calculated as 2-D integral/area or $\iint I(x,y)dA / \iint dA$.

The following is a description of the results, with reference to the appropriate figures, of the work described above. They clearly document structural and quantitative changes in the human glioma lines, including loss and gain of entire individual chromosomes and of chromosomal subregions. They also show that it has been possible to characterize both minor and predominant karyotypic features in each cell line. All chromosomes tested to date (i.e., 1, 4, 7, 18 and 22) clearly highlighted

numerical and/or structural aberrations, some of which were subtle.

Detection of numerical and structural chromosome aberrations in metaphase spreads.

05 Figures 6-8 and 12 show typical metaphase spreads from the malignant glioma cell lines TC 620 and 593 after CISS hybridization with biotinylated DNA inserts from each of the human chromosomes 1, 4, 7, 18 and 22. Hybridized inserts were detected with 10 avidin fluorescein isothiocyanate conjugates (FITC) and cells were counterstained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI). Chromosomes designated as "complete" had an apparently normal size, centromere index and DAPI 15 staining pattern. Despite this designation, these complete chromosomes may contain fine structural aberrations only detectable by additional investigations (see below). Apparently complete chromosomes 1, 4, 7, 18 and 22 were observed in both 20 TC 620 and TC 593. Additionally, other homologs of these chromosomes showed significant rearrangements and abnormalities, including translocations and deletions. The predominant numerical and structural aberrations delineated in each of these cell lines 25 are described below. A minimum of 25 good metaphase spreads were evaluated for each glioma line and for each chromosome. These data are summarized in Fig. 9.

Chromosome 1

30 In TC 620, the oligodendrogloma line, chromosome 1 inserts decorated two apparently

complete 1 chromosomes and two marker translocation chromosomes (Figs. 6A, 6B, 9). One marker was metacentric and contained an entirely decorated 1q arm, but its p arm was from another chromosome (of 05 unknown origin). The other marker chromosome was submetacentric and showed a small segment from another chromosome attached to the 1p arm. In both marker chromosomes breakpoints were localized close to the centromere in 1p11 or 1q11. The 10 identification of the 1p segment was established by DAPI banding (Fig. 6B), by 5'-bromo-2'-deoxyuridine (BrdU) banding, and by hybridization with a 1p36.3 probe (data not shown); the 1p36.3 probe 15 additionally revealed deletion of this subregion in one of the apparently complete 1 chromosomes. The overall picture was of a nearly trisomic representation of chromosome 1, with a common breakpoint, and subsequent translocation.

In TC 593, the glioblastoma line, an even more 20 complex pattern of numerical and structural chromosome 1 aberrations was observed. In a sample of 50 metaphase spreads, the majority (52%) showed six aberrant chromosomes that were decorated; 14% of the metaphases showed five aberrant chromosomes, and 25 34% showed higher numbers of chromosomes 1 segments (up to 14). Figures 6C, D and 9 show the most typical, predominant karyotype and demonstrates the rapid definition of chromosome 1 abnormalities in this cell line. Aberrations included three 30 acrocentric chromosomes with a consistent breakpoint in 1p1, chromosomes with a deletion of the distal

park of 1q, a submetacentric translocation chromosome with a loss of the complete 1q, and an iso(1p) marker chromosome (see Fig. 9).

Chromosome 4

05 In TC 620, chromosome 4-specific inserts decorated one apparently complete chromosome 4, and three additional chromosomes with segments containing chromosomes 4 DNA (Figs. 7F, 9). These latter segments on translocation chromosomes would

10 have been difficult to rapidly and unambiguously define with banding procedures alone. The smallest of the translocated chromosome 4 segments formed part of an approximately metacentric chromosome.

15 The two larger segments were found on submetacentric chromosomes of different overall size. In the smaller chromosome, the short arm and part of the long arm of 4 were present with an apparent breakpoint at 4q2, i.e., 4pter-4q2. In the larger submetacentric chromosome, a region that may

20 represent the rest of 4 (4q2-qter) appears. Thus the predominant karyotype of TC 620 showed only slightly more than two equivalents of chromosomes 4 (see also the area measurements described below). The non-4 regions have not been further defined.

25 In TC 593, there were generally only two chromosomes decorated by chromosomes 4 DNA inserts, and both of these were compatible with normal 4 chromosomes. Approximately 30% of the metaphase spreads in TC 593 showed an additional

30 submetacentric chromosome with chromosome 4 material

(Fig. 7E). Thus, although both 4 chromosomes were apparently normal, there was a significant under-representation of this chromosome in this pseudotetraploid line (Fig. 9).

05 Chromosome 7

Three complete 7 chromosomes, and one smaller metacentric chromosome containing translocated 7 material were typically found in TC 620 metaphase spreads (Figs. 8A, 9). The translocated chromosome 10 7 material included the short arm of chromosome 7 (as shown by DAPI banding; cf. Fig. 8B) and the pericentromeric heterochromatin with the breakpoint in 7q1 (see also below).

In TC 593, five chromosomes entirely decorated by chromosome 7 insert probes were regularly observed (Figs. 7G, 8E). Four of these appeared to represent complete number 7 chromosomes, whereas one was smaller and metacentric. DAPI banding (Fig. 8E, insert) and size measurement (cf. Fig. 13) were consistent with an iso(7p). This conclusion was further supported by double *in situ* hybridization experiments with biotinylated chromosome 7 inserts (detected with avidin FITC) and chromosome 7-specific alphoid AAF labeled sequences (detected with tetramethylrhodamine isothiocyanate (TRITC) conjugated second antibodies). They showed that only the four complete 7 chromosomes, contained a detectable 7 centromeric signal (metaphase, Fig. 7G,H; interphase, Fig. 7I, J). Thus, the iso(7p) 20 marker chromosome did not have a characteristic 25 30

-60-

centromeric region as it lacked both the 7 alphoid sequences and a small block of heterochromatin at 7q11 (see Fig. 8E, insert). In contrast, all four 7 chromosomes of TC 620 were labeled with the 7 05 alphoid probe (data not shown).

Chromosome 18

In TC 620, two apparently complete 18 chromosomes and a truncated minute chromosome were entirely decorated (Figs. 8C, D, 9). This truncated 10 chromosome is 18q- (and possibly also 18p-). The rest of the chromosome 18 region(s) was never detected.

Three translocation chromosomes involving 15 chromosome 18 material were typically detected, in addition to an apparently normal chromosome 18 in TC 593 metaphase spreads (Figs. 8F, G, 9). In a minor proportion of metaphases there was a small additional translocation observed. The exact chromosomal region from which this translocated 18 20 material derived could not be resolved by DAPI staining. The predominant karyotype for 18 is therefore close to tetrasomic in this cell line, but is under-represented in the pseudotriploid TC 620.

Both the 18q-marker chromosome in TC 620 and 25 the three translocated 18 chromosomes in TC 593 hybridized strongly to a chromosome 18-specific alphoid repeat. Accordingly, both intact and aberrant 18 chromosomes could also be counted after in situ hybridization with this centromeric probe.

30 Cremer, T. et al., Exp. Cell Res., 176:199-200

(1988) (see also below). DAPI banding and hybridization to 18-specific alphoid repeats indicated that these translocation chromosomes include the entire 18q region and the centromere, with breakpoints in
05 18p.

Chromosome 22

Two apparently normal 22 chromosomes were visualized in most TC 620 and TC 593 metaphase spreads (Fig. 10E). It was difficult to ascertain
10 small translocations of this chromosome since hybridization with chromosome 22 inserts resulted in some cross-hybridization to other chromosomes. Some of this cross-hybridization is probably due to shared sequences from the nucleolus organizer
15 regions (on five normal acrocentric human chromosomes) and to shared sequence motifs at the centromeres. McDermid, H.E. et al., Chromosoma, 94:228-234 (1986) and see Example 1. Finally, it should be noted that in contrast to conventional
20 banding analysis, the current experimental approach clearly delineates numerical and structural chromosome aberrations in metaphase spreads of very poor quality (Fig. 10G, H) or in early prophase nuclei (Fig. 10A). These preparations are not accessible
25 to banding analysis, as the chromosomes extensively overlie each other.

Evaluation of Chromosome Domains in Interphase Nuclei

One potential advantage of *in situ* methods is that individual human chromosomes may be directly visualized as discrete territories in interphase nuclei and thus can be of value in the analysis of solid tumor specimen. Manuelidis, L., Hum. Genet., 71:288-293 (1985); Schardin, M. *et al.*, Hum. Genet., 71:281-287 (1985); Pinkel, D. *et al.*, Proc. Natl. Acad. Sci. USA, 83:2934-2938 (1986). This feature of nuclear topography, also apparent in the malignant cells examined here (Figs. 7A-D, I, 10B-F), was evaluated for its accuracy and diagnostic usefulness. Figure 10A shows three apparently complete 7 chromosomes and one translocated 7p arm in a prophase TC 620 nucleus. Figures 3, and 10B show five chromosome 7 domains in interphase nuclei of TC 593, as previously depicted in metaphase spreads. Figure 10C shows a TC 620 interphase nucleus with two 18 domains of comparable sizes to those seen in normal diploid nuclei (see Example 1). A third, appreciably smaller, decorated 18 domain was also detected and represents the truncated 18 chromosomes seen in metaphase spreads described above. Figure 10D shows four chromosome 18 domains in an interphase nucleus of TC 593, which again is comparable to the numbers in metaphase nuclei. Figure 10E shows a TC 620 interphase nucleus with four chromosomes 1 domains, while Figure 10F shows a TC 593 nucleus with at least five separate chromosome 1 domains (compare Fig. 6A, B and C,D, respectively).

While the hybridization patterns of nuclei shown in Figure 10 were highly characteristic for each cell line, counts of interphase chromosome domains have some inherent difficulties. As an 05 example, Fig. 11A (dark columns) presents an analysis of the counts of labeled interphase domains in randomly selected nuclei of diploid human lymphocytes hybridized with 7 library inserts. Although the number and relative size of chromosome 10 specific domains can be accurately assessed in the majority of nuclei, not all nuclei present a reliable index of the chromosomal constitution, since a considerable fraction of nuclei reveal only one decorated domain and occasional nuclei show no 15 signals. Furthermore all domains are not always cleared separable in these 2-D preparations.

Figure 11 shows representative counts of these preparations. In agreement with TC 593 metaphase counts of chromosome 4, nuclear counts generally 20 showed two clearly separated domains (Fig. 11E). However, the percentage of two-signal preparations was smaller in interphase than in metaphase (45.3% vs. 64%). This artifactual decrease was largely due to a corresponding increased percentage of nuclei 25 showing only one decorated domain or no signal at all. Counts of zero or one domain were not present in metaphase spreads. Significantly, 19.3% of the interphase TC 593 nuclei displayed three clearly separated chromosome 4 domains, and these extra 30 domains were not present in interphase nuclei of diploid human lymphocytes hybridized to this or

other libraries under the same conditions (Fig. 10A; Example 1). Finally, the ratio of two versus three domains was identical for both metaphase and interphase cells. Thus interphase nuclei can be reliably 05 used for the detection of extra copies of a single chromosome or chromosomal segment but have limited reliability for detecting the loss of chromosome copies.

In situ hybridization of probes from subregions 10 of interphase chromosomes may more accurately reflect general counts of chromosomal constitution than library probes (Fig. 11A), provided they are done under appropriately high stringency conditions Rappold, G. et al., Hum. Genet., 67:317-325 (1984); 15 Cremer, T. et al., Hum. Genet., 74:346-352 (1986); Cremer, T. et al., Exp. Cell Res., 176:199-220 (1988). However, such regional segment probes do not delineate translocated elements or aberrant chromosomes that lack this segment. Therefore such 20 probes are also not entirely accurate. For example, counts of chromosomes 1 in TC 620 and TC 593 with a probe specific for 1ql2 indicated fewer 1 chromosomes than shown here with CISS hybridization (Fig. 9). Cremer, T. et al., Exp. Cell Res., 176:199-220 25 (1988). Counts of chromosome 7 using only a centromeric sequence further emphasize this point (see above). Double in situ hybridization with the AAF-modified 7 alphoid probe and biotinylated chromosome 7 library inserts typically showed 30 interphase nuclei with five domains, of which only four were simultaneously labeled by the centromeric

probe (Figs. 7I, J, 11C). In TC 620, however, both probes gave identical results (Fig. 11B).

Over Representation and Under Representation of Specific Chromosomes

05 The relative chromosomal dosage in these glioma lines, was also assessed with particular interest in chromosome 7, which has been noted to be generally over-represented in gliomas. Bigner, S.H. *et al.*, *Cancer Genet. Cytogenet.* 29:165-170 (1986); Shapiro, 10 J.R., *Semin. Oncol.*, 13:4-15 (1986). For comparison, other individual chromosome probes were used as controls. Metaphase chromosomes counts have shown that TC 620 is pseudotriloid with a modal number of 64 chromosomes, while TC 593 is pseudotetraploid 15 with a modal number of 83. Manuelidis, L. and Manuelidis, E.E., *IN: Progress in Neuropathology*, Vol. 4, pp 235-266, Raven Press, N.Y. (1979). Accordingly, a chromosome and its segments together would be present in a balanced state if three 20 complete copies were present in TC 620, and four in TC 593.

A relative over-representation is present if more than these respective copy numbers can be demonstrated. A number lower than the expected 25 (trisomic or tetrasomic) value indicates that the chromosome is relatively under-represented in the karyotype. In cases where additional DAPI banding information was sufficient to define the selectively decorated abnormal chromosome, the chromosome pieces 30 labeled by the chromosome-specific inserts were put

-66-

together for analysis (Fig. 9). In the second approach, computer analyses were used to independently verify these results (see below).

TC 620 analyzed by banding showed the 05 equivalent of three 1 chromosome and thus indicated a balanced state for this chromosome. The same was true for the 1p arm in TC 593 which was present in four copies. However, the distal part of 1q was under-represented in TC 593 (see the detailed 10 description given above). In both glioma lines, 7q appeared to be balanced, while 7p was over-represented once in TC 620 and twice in TC 593. Additionally, in both glioma lines chromosomes 22 was clearly under-represented. In order to confirm 15 this finding, double *in situ* hybridization with inserts of chromosomes 7 and 22 was performed. An example of this is shown in Fig. 12 and demonstrates over-representation of 7 DNA and under-representation of 22 DNA in the same cell. Metaphase counts 20 done in both cell lines by this method of analysis are depicted for chromosome 7 in Fig. 11B, C (dark column) and for chromosome 22 in Fig. 11E. In summary, these two gliomas both show relative under-representation of chromosome 22 and over- 25 representation of the 7p arm. The significant under-representation of chromosome 4 in TC 593, and a portion of 4 in TC 620 is also notable.

Digitized images were also used to quantitatively measure decorated areas in metaphase 30 preparations and in interphase cells where chromosomal domains were well resolved.

Quantitative evaluation of chromosome equivalents (Table 3) indicated highly concordant numbers for interphase versus metaphase in 5 of 6 examples; only in TC 593 decorated with 18 inserts was there a
05 discrepancy. This may be due to the small sample size.

Table 3.

Twenty-four metaphase spreads showing the predominant number of chromosomes decorated with DNA 10 inserts from libraries of chromosomes 4, 7 and 18 were compared to twenty-eight interphase nuclei with well-separated domains using the same probes. Images were taken under identical (linear film) 15 conditions and digitized. In each metaphase spread, areas obtained for each normal and aberrant chromosome were divided by the mean area obtained for n 20 apparently complete chromosomes. In interphase nuclei, domains were compared assuming that the largest n-labeled domains represented complete (normal) interphase chromosomes. Thus the sum of 25 these normalized values represents a measure of the number of specific chromosomes equivalents in a single cell. The mean values of several cells are shown for each case. The mean numbers of chromosomes equivalents obtained for interphase and metaphase cells show a strong overall correlation coefficient of $r = + 0.95$. Compared with area measurements, the mean numbers of chromosomes equivalents determined by 2-D intensity integrals

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(See above) showed an overall correlation coefficient of $r = + 0.99$.

05 Table 3. Mean number of chromosome equivalents measured by digital image analysis in malignant glioma cell lines after CISS hybridization

	Chromosome	Cell Line	Interphase	Chromosome equivalents	Metaphase	Expected
10	4	TC593	2.0		2.0	4.0
	10	TC620	2.5		2.4	3.0
	7	TC593	4.4		4.6	4.0
		TC620	3.5		3.3	3.0
15	18	TC593	3.0		3.6	4.0
		TC620	2.5		2.3	3.0

15 Chromosome equivalents derived from digital image analysis independently confirm the relative representation of target chromosomes noted in both glioma lines by DAPI banding. The segments that comprise the total metaphase signal are further 20 detailed graphically in Fig. 13. Computer analysis was especially useful in cases where the breakpoints involved in translocated segments could not be unambiguously defined. They were also of value in a 25 quantitative assessment of interphase-metaphase correlations, and of normal and aberrant chromosomes with distinctly different sizes.

EXAMPLE 3 Rapid Detection of Human Chromosome 21
Aberrations By In Situ Hybridization

DNA Probes

All plasmids contain inserts of human
05 chromosome 21 that were mapped to 21q22.3. Moisan,
J.P., Mattei, M.G., Baeteman-Volkel, M.A., Mattei,
J.F., Brown, A.M.C., Garnier, J.M., Jeltsch, J.M.,
Masiakowsky, P., Roberts, M. & Mandel, J.L. (1985)
Cytogenet. Cell Genet. 40, 701-702 (abstr.). Tanzi,
10 Watkins, P., Gibons, K., Faryniarz, A., Wallace,
M., Hallewell, R., Conneally, P.M. & Gusella, J.
(1985) Cytogenet. Cell Genet. 40, 760 (abstr.). Van
Keuren, M.L., Watkins, P.C., Drabkin, H.A., Jabs,
E.W., Gusella, J.F. & Patterson, D. (1986) Am. J.
15 Hum. Genet. 38, 793-804. Nakai, H., Byers, M.G.,
Watkins, P.C., Watkins, P.A. & Shows, T.B. (1987)
Cytogenet. Cell Genet. 46, 667 (abstr.). Munke, M.,
Foellmer, B., Watkins, P.C., Cowan, J.M., Carroll,
A.J., Gusella, J.F. & Fracke, U. (1988) Am. J. Humm.
20 Genet. 42, 542-549. All inserts were either known
or verified by Southern blot analysis to be
single-copy DNA: the plasmids other than pS2 are
subclones derived from a λ phage library or a cosmid
library. Van Keuren, M.L., Watkins, P.C., Drabkin,
25 H.A., Jabs, E.W., Gusella, J.F. & Patterson, D.
(1986) Am. J. Hum. Genet. 38, 793-804. Masiakowski,
P., Breathnach, R., Bloch, J., Gannon, F., Krust, A.
& Chambon, P. (1982) Nucleic Acids. Res. 10,
7895-7903. Watkins, P.C., Tanzi, R.E. Gibbons,
30 K.T., Tricoli, J.V., Landes, G., Eddy, R., Shows,

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T.B. & Gusella, J.F. (1985) Nucleic Acids Res. 13, 6075-6088. Watkins, P.C. Watkins, P.A., Hoffman, N.

& Stanislovitis, P. (1985) Cytogenet. Cell Genet.

40; 773-774 (abstr.). The plasmids are listed in

05 Table 4 with the Human Gene Mapping Workshop symbols and the approximate insert fragment length. Kaplan, J.C. & Carrit, B. (1987) Cytogenet. Cell Genet. 46, 257-276.

10 Table 4. Plasmids with inserts from 21q22.3

	Plasmid	Insert length	Plasmid	Insert length		
				Plasmid		
		kb		kb		
	BCEI	pS2 (23)	0.6	D21S56	pPW520-10R	4.6
15	D21S3	pPW231F	0.8		pPW520-11R	1.8
		pPW231G	0.7	D21S57	pPW523-10B	6.5
	D21S23	pPW244D	1.0		pPW523-1H	7.0
	D21S53	pPW512-6B	3.0		pPW523-5R	2.2
		pPW512-8B	3.8		pPW523-10R	3.8
20		pPW512-1H	2.9		pPW523-19R	2.5
		pPW512-16P	2.7	D21S64	pPW551-8P	1.9
		pPW512-18P	1.6		pPW551-12P	4.2
		pPW512-4R	4.7	D21S71	pPW519-10P	0.8
		pPW512-12R	2.0		pPW519-11P	3.0
25	D21S55	pPW518-4H	1.6		pPW519-1R	6.0
		pPW518-10P	2.9		pPW519-8R	2.9
		pPW518-5R	5.2		pPW519-9R	1.7
	D21S56	pPW520-5B	5.0		pPW519-14R	4.0
		pPW520-6B	1.0		pPW519-22R	1.8

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Preparation of plasmid DNA was according to standard protocols. Maniatis, T., Fritsch, E.F. & Sambrook, J. (1982) Molecular Cloning: A Laboratory manual (Cold Spring Harbor Lab., Cold Spring Harbor, NY). Various probe sets were obtained by pooling plasmids (equal molar amounts), resulting in DNA probe complexities of 95 kb (all plasmids listed). 75 kb (plasmids labeled with an asterisk), or 29 kb (plasmids labeled with a dagger).

05 10 The human chromosome 21 genomic library LL21NS02 was obtained from the American Type Culture Collection and amplified on agar plates as recommended. Phage DNA was prepared and digested with HindIII, and the DNA inserts were separated from the vector arms by preparative gel electrophoresis in 0.6% agarose. DNA was isolated from gel slices by electroelution; purified by Elutip-d chromatography. (Schleicher & Schuell); extracted with phenol/chloroform, 1:1 (vol/vol); and precipitated with ethanol.

15 20

Human Cells

Metaphase spreads and interphase nuclei were prepared from (i) lymphocyte cultures of normal (46, XY) individuals, (ii) lymphocytes of Down syndrome (47, +21) individuals, (iii) chorionic villi samples cultured for prenatal diagnosis (ii and iii were provided by T. Yan-Geng, Yale University Cytogenetics Laboratory), and (iv) cultures of

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TC620, an oligodendrogloma-derived pseudotriploid cell line. Manuelidis, L. & Manuelidis, E.E. (1979) in *Progress in Neuropathology*, ed. Zimmerman, H.M. (Raven, Press New York), Vol. 4, pp. 235-266.

05 Standard techniques of colcemid treatment, hypotonic treatment, and methanol/acetic acid fixation were used. Biopsy material from the cortical region of a "normal" human brain (46, XX) was fixed, sectioned, and permeabilized as described. Manuelidis, L. &

10 Borden, J., Chromosoma, 96:397-410(1988).

In situ Hybridization

Various combinations of plasmid DNA, labeled with Bioll-dUTP by nick-translation, were used for hybridization at concentrations ranging from 2 to 15 µg/ml depending on the pool size. Brigati, D.J., Myerson, D. Leary, J.J., Spalholz, B., Travis, S.Z., Fong, C.K.Y. Hsiung, G.D. & Ward, D.C. (1983) *Virology* 126,32-50. For example, 15 µg/ml was used when the probe mixture contained 94 kilobases (kb)

20 of insert DNA; the probe concentration was decreased in proportion to the sequence complexity of the probe mixture. The size of the probe DNA was adjusted to a length of 150-250 nucleotides empirically by varying the DNase concentration in

25 the nick-translation reaction. The hybridization cocktail also contained 50% formamide, 0.30 M NaCl, 0.03 M sodium citrate (pH7), 10% (wt/vol) dextran sulfate, and on occasion 0.5 mg of sonicated salmon sperm DNA per ml. Simultaneous denaturation of

30 probe and target DNA was carried out at 75°C for 6

min (metaphase spreads) or 94°C for 11 min (tissue slices). Hybridization reactions were incubated at 37°C overnight.

Delineation of individual chromosomes with DNA

05 probes derived from sorted human chromosomes was done by (CISS) hybridization as described above. Briefly, biotinylated chromosome 21 library DNA inserts (5 μ g/ml), DNase-digested human genomic DNA (200 μ g/ml), and salmon sperm DNA (800 μ g/ml) were

10 combined in the hybridization solution, heat-denatured, and partially prehybridized for 10-30 min at 37°C before application to a separately denatured specimen.

Posthybridization washes, detection of

15 hybridized probe by using either alkaline phosphate-conjugated avidin or fluorescein isothiocyanate-conjugated avidin, and photographic conditions were as described in Example 1. When probe sets containing 29 kb or less of target

20 sequence were used, the fluorescein isothiocyanate detection was generally enhanced by one cycle of signal amplification as described in Example 1.

All quantitative analyses of interphase signals were carried out by using slides from several

25 independent experiments, with more than 100 nuclei being analyzed per slide. Comparison of signals in normal and trisomic samples was done in a blind-study fashion.

This work demonstrated that CISS hybridization,

30 under the conditions described, resulted in rapid detection of numerical and structural aberrations of

chromosome 21 in both metaphase and interphase cells.

Use of Cloned DNA Fragments From Human Chromosome 21 to Specifically Label Chromosomes in Lymphocyte

05 Metaphase Spreads and Interphase Nuclei

The maximal amount of unique sequence DNA in the probe set was ca94 kb; this probe set resulted in a clearly visible labeling of the terminal region of both chromatids of the chromosome 21 homologs (see Fig. 14B). These signals were seen unambiguously and without exception in all metaphase spreads, even in spreads of poor quality or from prophase cells (not shown). In normal interphase cells, the majority (65-75%) of nuclei exhibited two signals (see Fig. 14C), 25-30% showed one signal, and less than 5% showed no signal. Nuclei with three signals were found only rarely (<0.2%) and may reflect incomplete hybridization to a few tetraploid cells in the sample. Similar results were obtained 10 with probe sets containing 29 or 75 kb of DNA. With probe sets containing fewer than 20 kb of insert DNA, there were increased numbers of cells with less than two signals. Thus, these probe sets were deemed unsuitable for diagnostic purposes. However, 15 20 25 such probes still yielded specific signals on the majority of chromosomes 21, even with a 6-kb single-copy DNA (see Fig. 14A), especially when signal amplification was used.

Use of chromosome library DNA CISS hybridization for
detecting chromosome 21

Chromosome 21 was specifically and entirely decorated in normal lymphocyte metaphase spreads,
05 although some additional minor binding sites were seen at or near the centromeric region of other acrocentric chromosomes, especially chromosome 13 (normal karyotype not shown; Fig. 14F). Suppression with additional DNA including a plasmid L1.26, which
10 detects a repetitive DNA located predominantly at the centromeric region of chromosomes 13 and 21, did not efficiently suppress the minor non-21 chromosomal signals. Devilee, P., Cremer, T., Slagboom, P., Bakker, E., Schoil, H.P., Hager, H.D.
15 Stevenson, A.F.G., Cornelisse, C.J. & Pearson, P.L. (1986) Cytogenet. Cell Genet. 41,193-201.
Quantitative evaluation of interphase nuclei signals again showed a negligible portion of nuclei with three signals; however, a significant increase in
20 nuclei with less than two signals was observed (50-60% with two signals, 35-45% with one signal, and 5-10% without a signal). The numerical differences observed with the two different probes can be explained in part by the number of nuclei (up
25 to one of three) that were excluded from the latter analysis because they exhibited larger and more diffuse signals, most likely from more than one chromosome that could not be resolved unambiguously as two separate chromosome domains in a
30 two-dimensional representation. The minor cross-hybridizing sites noted above presented a

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second experimental complication but did not adversely influence data interpretation.

Testing of Cells Containing Chromosome 21

Aberrations

05 The optimal (94 kb) plasmid pool as well as CISS hybridization with chromosome 21 library inserts were tested further by using cells containing chromosome 21 aberrations. Both probe sets permitted a fast and unambiguous diagnosis of 10 trisomy 21 in all metaphase spreads from Down syndrome lymphocyte cultures (see examples in Fig. 14 D and E). Furthermore, the quantitative distribution of hybridization signals in interphase nuclei of the same preparation, analyzed as 15 described above, was similar with either type of probe [$<5\%$ of cells with no signal, 5-15% with one signal, 25-35% with two signals, and 55-65% with three signals (Fig. 14 F-J)]. Although the library DNA inserts gave up to 15% of four-signal nuclei 20 (compare Fig. 14 F and G), most likely due to the minor binding sites on other chromosomes, the plasmid pool revealed only a negligible percentage of nuclei ($<0.2\%$) with four signals. These results indicate that trisomy 21 can be detected in a 25 diagnostically meaningful way with small populations of nonmitotic cells.

Localization of Chromosome 21 DNA in Embryonic Chorionic Villi Cells

Embryonic chorionic villi (CV) cells were also investigated with the 94 kb plasmid probe sets in a 05 case where the father had a reciprocal t(4:21) translocation. Hybridization to metaphase spreads of the CV cells showed that the translocated chromosome (4pter→4q33::21q11.2→21qter) was indeed inherited by the fetus (see Fig. 13 L and M). The 10 signals in the interphase cell nuclei (see Fig. 14K) of the CV cells had a distribution that paralleled that of cells with a normal karyotype (see above), indicating a balanced representation of 21q22.3 and excluding Down 15 syndrome as a possible diagnosis. A small increase of nuclei with three and four signals (both <5%) over that of normal lymphocytes was also observed, probably reflecting a higher portion of tetraploid cells in such CV samples.

20 Localization of Chromosome 21 DNA in of Glioma Tumor Cells

The diagnostic potential of the chromosome 21 probes was further tested by using a glioma tumor cell line, TC620, known to be pseudotriploid with a 25 highly rearranged genome. Cremer, T. et al., Exp. Cell Res., 176:199-220 (1988); Cremer, T. et al., Hum. Genet., In Press, (1988); Manuelidis, L. and E.E. Manuelidis, In: Progress in Neuropathology, 4:235-266 (ed. Zimmerman, H.M.) (1979). The 30 metaphase spreads revealed two apparently normal

chromosomes 21 and one translocation chromosome (see Fig. 14 N and O). Interestingly, the chromosome 21 DNA on the translocation chromosome labeled by the library probe has a size equivalent to a normal 21q region, thus suggesting a Robertsonian translocation event. However, fine structural aberrations of 21q (i.e., small deletions, etc.) cannot be excluded by this analysis. The interphase signals seen with both the plasmid probe set and the library inserts were consistent with trisomy 21q22.3 and trisomy 21, respectively.

Localization of Chromosome 21 in DNA Sequences in Solid Tissues

The ability of the 94 kb plasmid probe set to 15 localize chromosome 21 DNA sequences in solid tissues was also assessed. Both chromosomes 21 were clearly labeled by the probe, and located near the nucleolus; this nuclear location is consistent with the fact that chromosome 21 contains a ribosomal 20 gene cluster that is usually localized in the nucleolus. This observation suggests that these probes may also prove useful for evaluating the frequency of chromosome 21 mosaicism in specific cell or tissue types. In addition, it should be of 25 interest to see if the various karyotypic changes associated with the Down syndrome phenotype alter the normal nuclear topography of chromosome 21 in neuronal tissue.

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Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific 05 embodiments of the invention described specifically herein. Such equivalents are intended to be encompassed in the scope of the following claims.

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Claims

1. A method of labeling individual mammalian chromosomes in mitotic cells or in interphase cells by *in situ* hybridization with 05 chromosome-specific probes, to produce a chromosome-specific signal.
2. A method of labeling individual human chromosomes in mitotic cells or in interphase cells by *in situ* hybridization with 10 chromosome-specific probes, to produce a chromosome-specific signal.
3. A method of producing highly specific decoration of an individual human target chromosome, comprising *in situ* suppression 15 hybridization of labeled DNA probes, which are chromosome specific, to DNA in human mitotic cells or in human interphase cell.
4. A method of Claim 3, wherein the DNA probes are selected from the group consisting of total 20 recombinant library DNA, DNA inserts purified from a chromosome-derived recombinant DNA library, and specific DNA fragments derived from chromosomes.
5. A method of Claim 4, wherein the labeled DNA 25 probes are selected from the group consisting of: DNA probes labeled with at least one

fluorochrome; DNA probes labeled with at least one member of a specific binding pair; and DNA probes labeled with an enzyme.

6. A method of Claim 5, wherein the fluorochrome
05 is selected from the group consisting of fluorescein, rhodamine, Texas red, Lucifer yellow, phycobiliproteins and cyanin dyes and the member of a specific binding pair is biotin.
- 10 7. A method of assessing chromosome aberrations in human cells by chromosomal in situ suppression hybridization.
8. A method of Claim 7, wherein the human cells
15 are selected from the group consisting of metaphase cells, prophase cells and interphase cells.
9. A method of detecting chromosome aberrations in human aneuploid cells, comprising
 - a) combining
 - 20 1) the human aneuploid cells, treated so as to render nucleic acid sequences present available for hybridization with complementary nucleic acid sequences; and
 - 2) a hybridization mixture comprising
 - 25 labeled human DNA derived from a specific chromosome; competitor DNA; and nonhuman genomic DNA, under conditions appropriate

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for hybridization of complementary nucleic acid sequences to occur; and

05 b) detecting labeled human DNA derived from the specific chromosome hybridized to nucleic acid sequences from the aneuploid cells.

10. 10. A method of Claim 9, wherein the aneuploid cells are human tumor cells.

11. 11. A method of Claim 10, in which the human tumor cells are selected from the group consisting 10 of: metaphase cells, prophase cells and interphase cells.

12. 12. A method of Claim 10, wherein the human tumor cell is a human glioma cell.

13. 13. A method of detecting in a sample numerical 15 alterations in a human chromosome present in the sample, comprising:

14. a) combining

15. 1) the sample, treated so as to render 20 nucleic acid sequences present in the sample available for hybridization with complementary nucleic acid sequences; and

16. 2) a hybridization mixture comprising 25 labeled human DNA derived from the selected chromosome; competitor DNA; and nonhuman genomic DNA, under conditions appropriate for hybridization of

complementary nucleic acid sequences to occur; and

05 b) detecting labeled DNA derived from the selected chromosome hybridized to nucleic acid sequences present in the sample.

14. A method of Claim 13, wherein the selected human chromosome is selected from the group consisting of the following chromosomes: 13, 18, 21, X and Y.

10 15. A method of Claim 13, wherein the selected human chromosome is chromosome number 21 and the labeled human DNA derived from the selected chromosome is DNA inserts purified from a chromosome-derived recombinant DNA library.

15 16. A method of determining over-representation or under-representation of a selected chromosome or a portion thereof in human tumor cells, comprising the steps of:

20 a) combining

1) human tumor cells, treated so as to render nucleic acid sequences present in the cells available for hybridization with complementary nucleic acid sequences; and

2) a hybridization mixture comprising

25 labeled DNA fragments derived from a selected chromosome; competitor DNA; and nonhuman genomic DNA, under conditions appropriate for hybridization of

complementary nucleic acid sequences to occur; and

05 b) detecting labeled human chromosome-specific DNA fragments hybridized to nucleic acid sequences from the tumor cells.

17. A method of identifying chromosome-specific DNA present in a selected mammalian chromosome, comprising:

10 a) combining the following substances:

1) the selected mammalian chromosome;

2) DNA fragments derived from the selected mammalian chromosome bearing a detectable label;

3) competitor DNA; and

15 4) carrier DNA,
under conditions appropriate for hybridization of complementary nucleic acid sequences to occur; to form a complex of the DNA fragments bearing a detectable label with the selected mammalian chromosome; and

20 b) detecting complexes formed in step (a).

25 18. A method of Claim 17, further comprising isolation of chromosome-specific DNA in a selected mammalian chromosome by separating the selected complexes formed from the remaining substances combined in step (a).

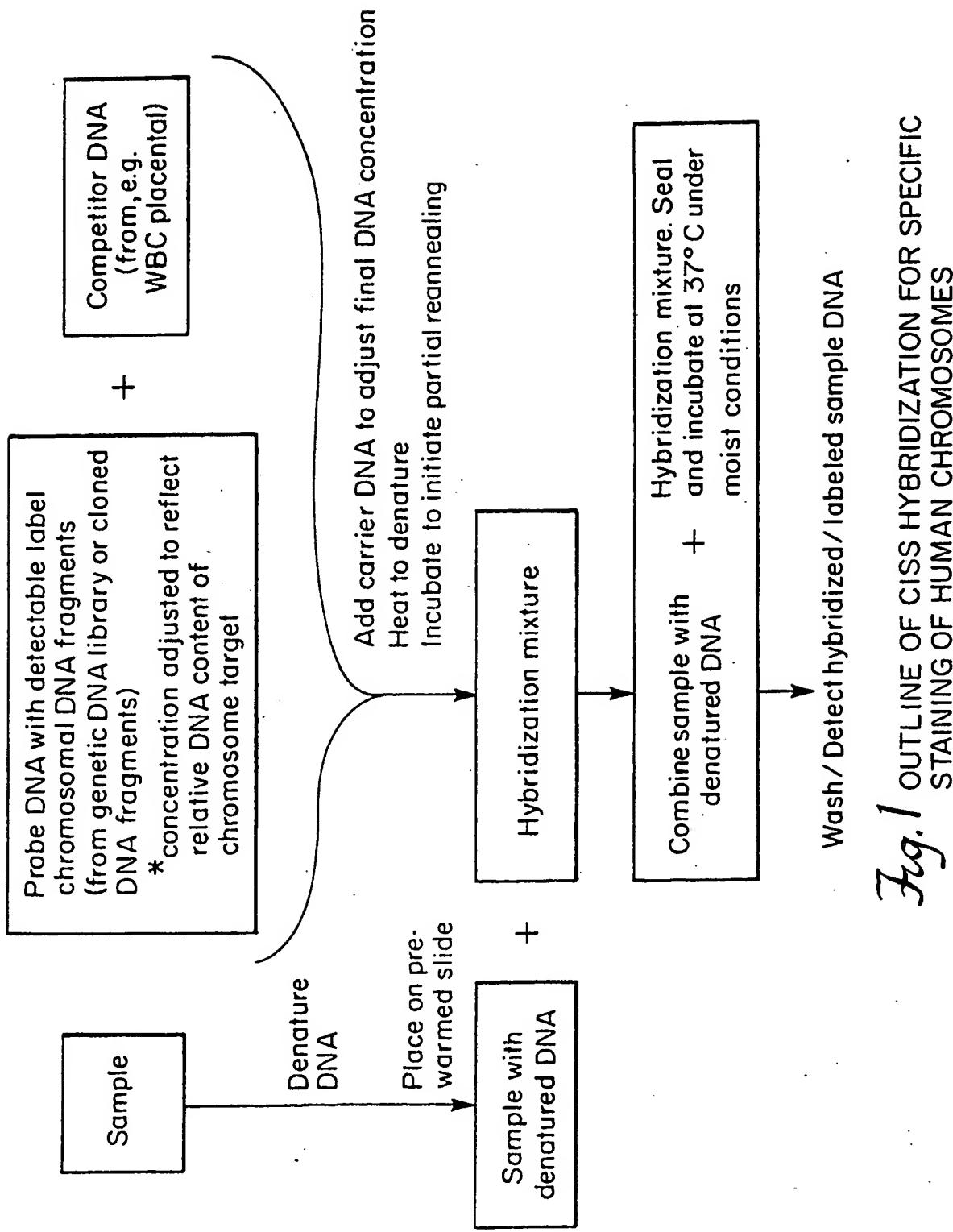
19. A method of identifying a selected chromosome in mammalian metaphase cells or mammalian interphase cells, comprising combining mammalian metaphase cells or mammalian interphase cells, said cells previously treated to render DNA present available for hybridization with complementary nucleic acid sequences, with two or more selected detectably labeled chromosome-specific nucleotide probes of high genetic complexity, under CISS-hybridization conditions to label selected chromosomes and detecting labeled selected chromosomes.

20. A method of identifying at least two different selected chromosomes in mammalian metaphase cells or mammalian interphase cells, comprising combining mammalian metaphase cells or mammalian interphase cells, said cells previously treated to render DNA present available for hybridization with complementary nucleic acid sequences, with a set of detectably labeled chromosome-specific probes, said set comprising at least a first detectably labeled nucleic acid probe complementary to one of the different chromosomes to be identified and a second detectably labeled nucleic acid probe complementary to the second of the different chromosomes to be identified, the detectable label on the first detectably labeled nucleic acid probe being different from

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the detectable label on the second detectably labeled nucleic acid probe, under CISS-hybridization conditions, to produce detectably-labeled selected chromosomes, and detecting
05 labeled selected chromosomes.

21. The method of Claim 20 wherein the detectably labeled chromosome-specific probes are fluorescently labeled.

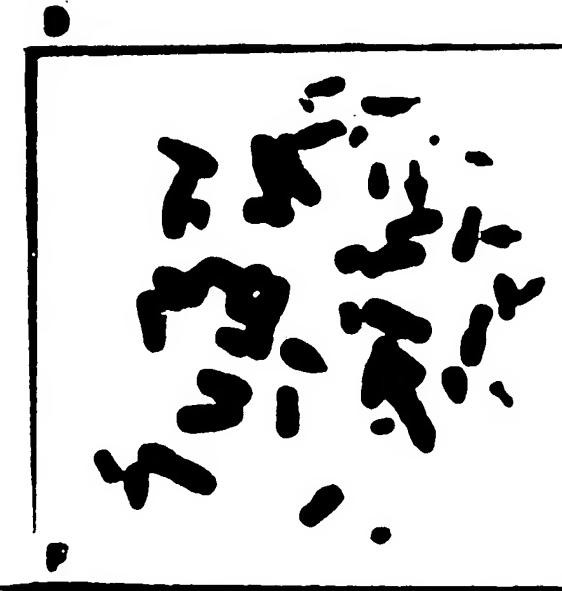


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Fig. 1 OUTLINE OF CISS HYBRIDIZATION FOR SPECIFIC STAINING OF HUMAN CHROMOSOMES

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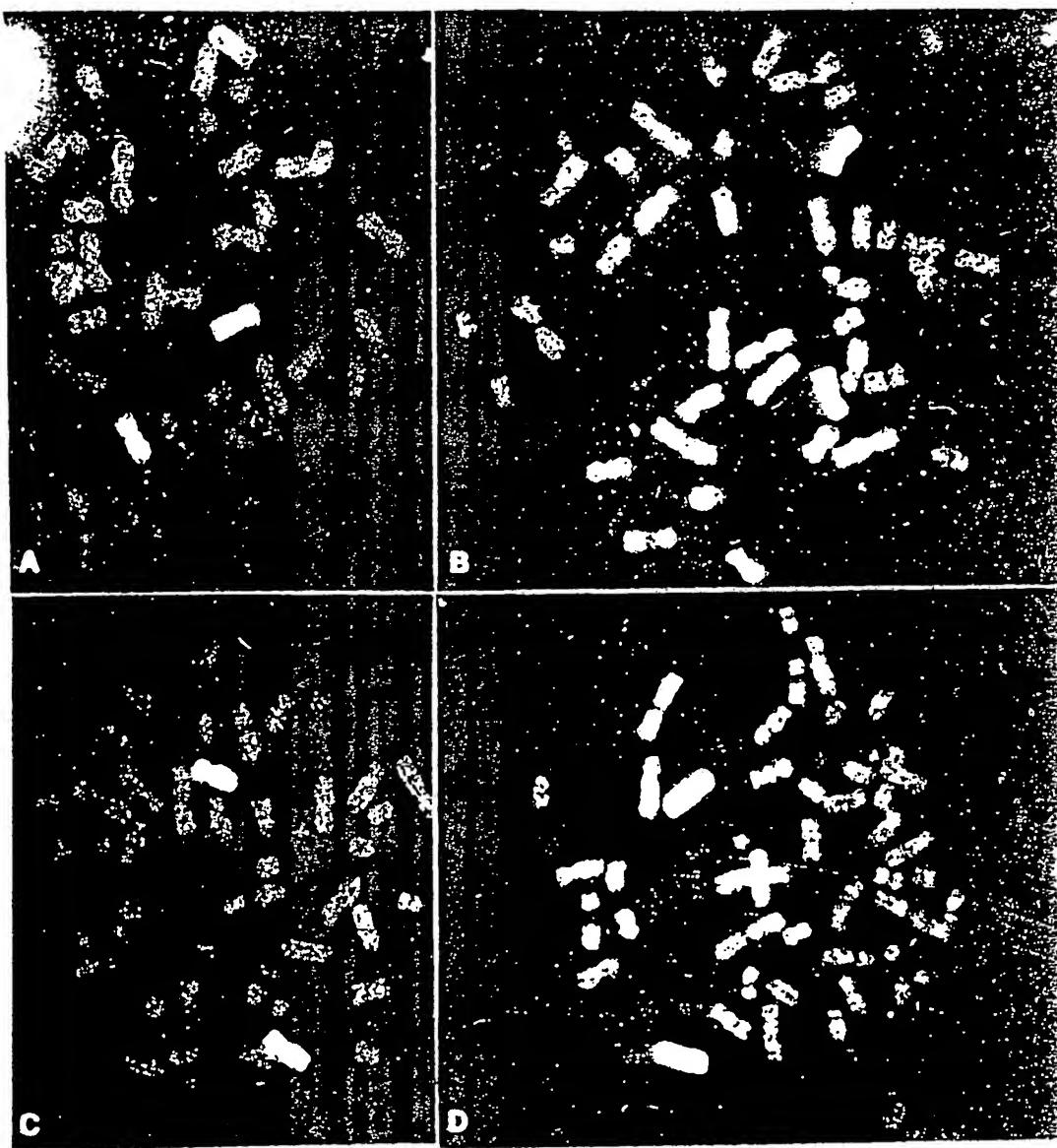
FIG.2



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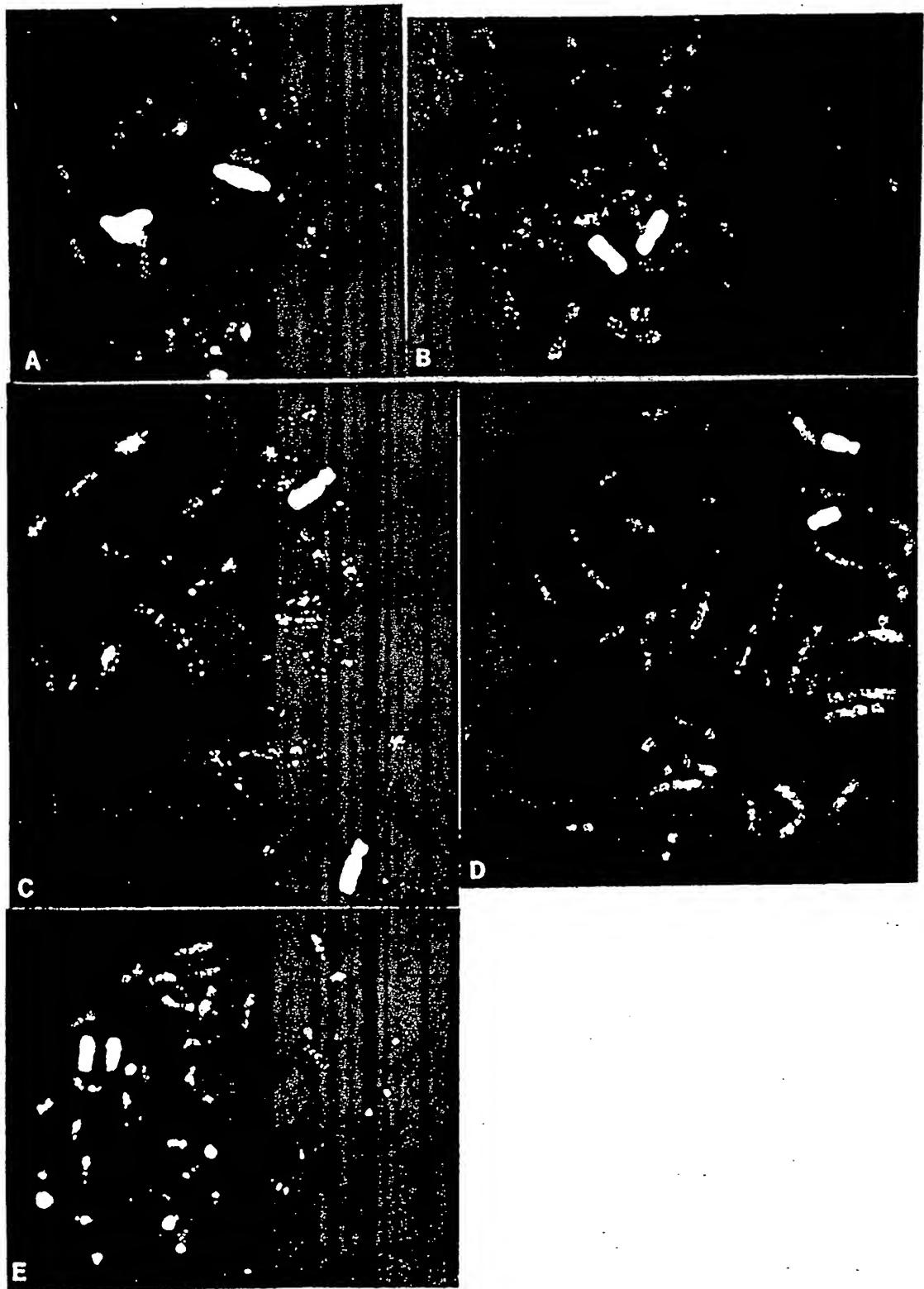
FIG.3



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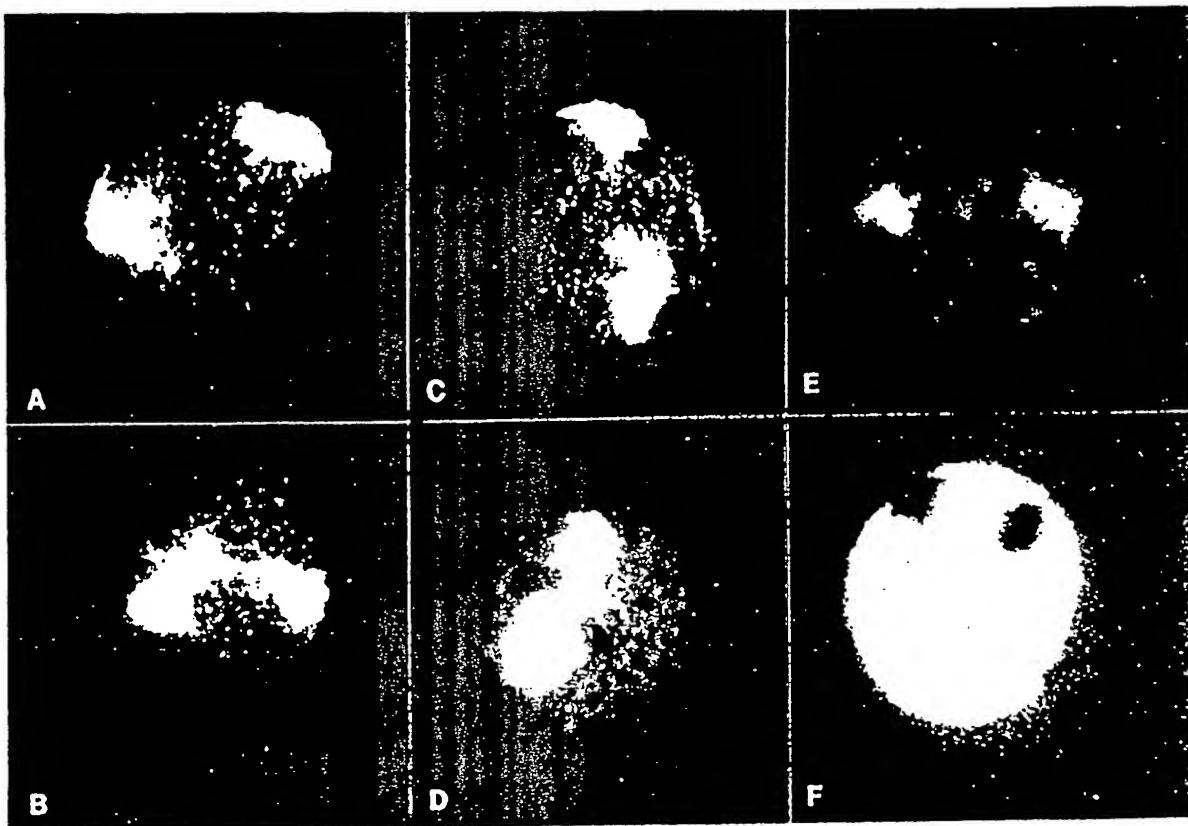
4/14

FIG.4



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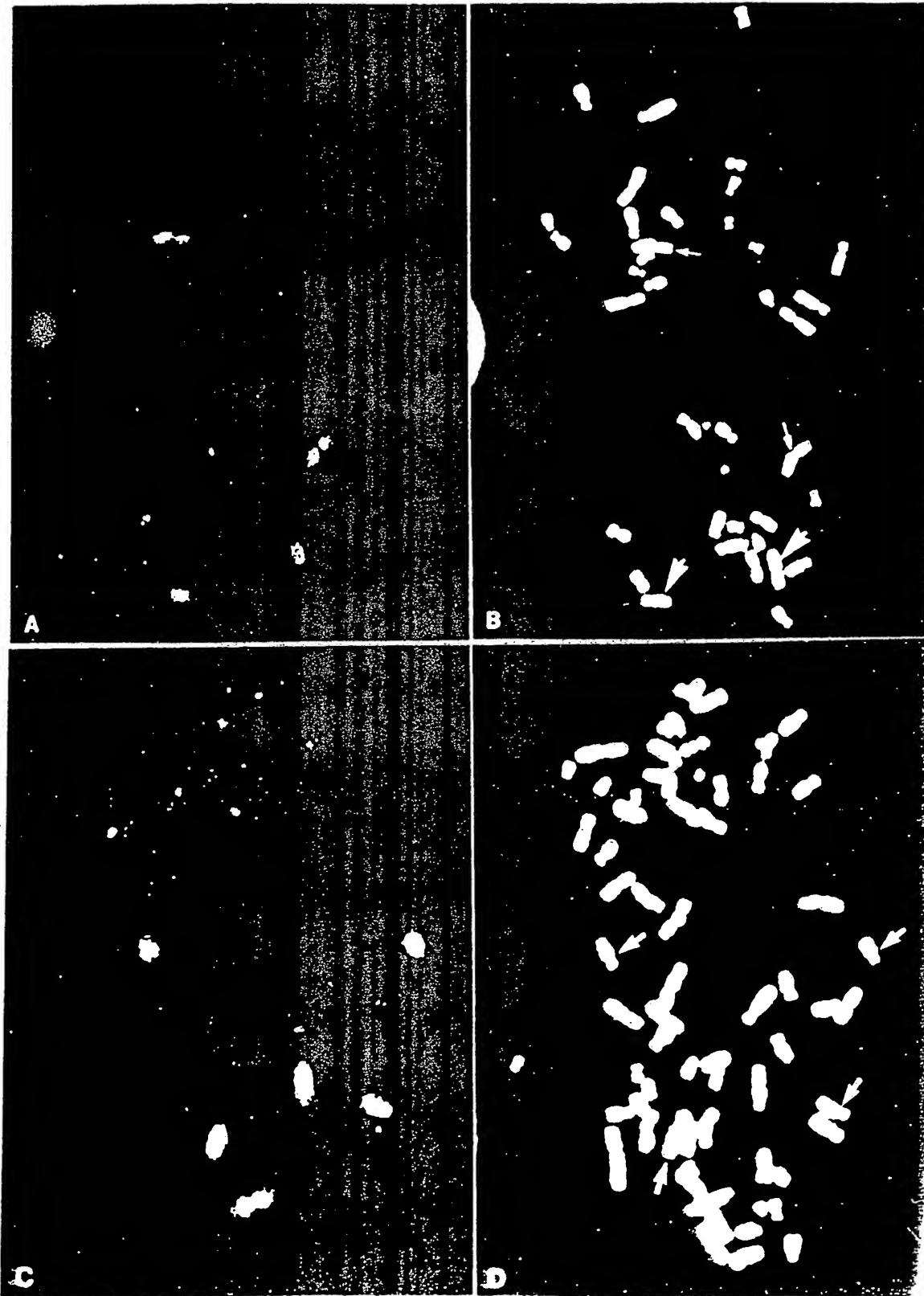
FIG.5



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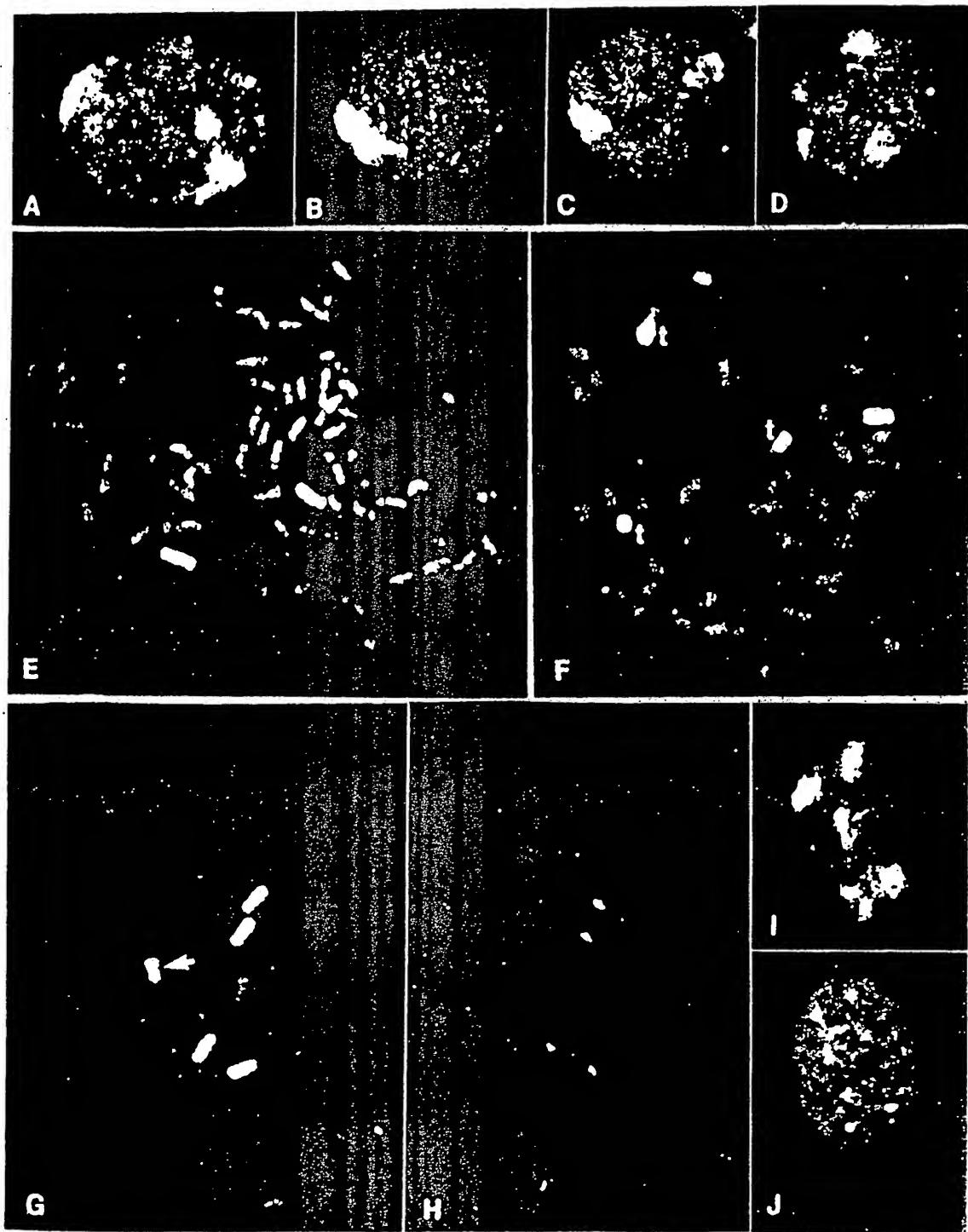
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FIG.6



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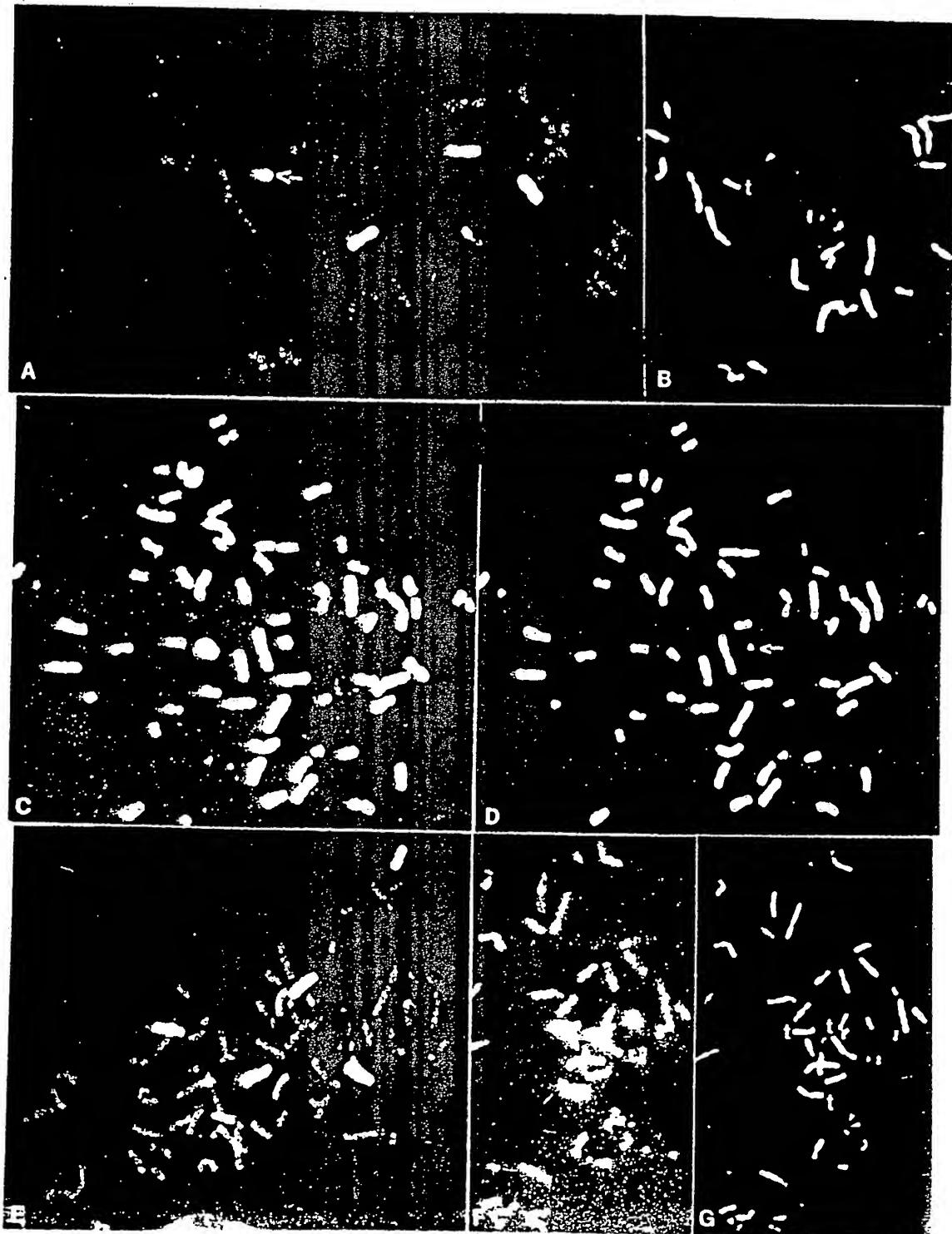
FIG.7



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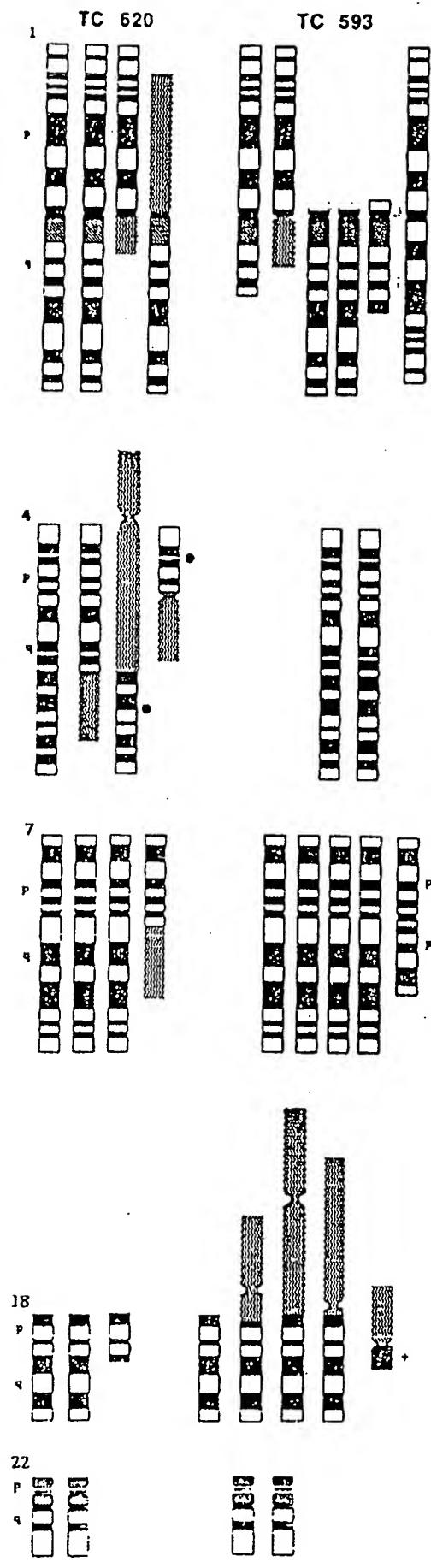
FIG.8



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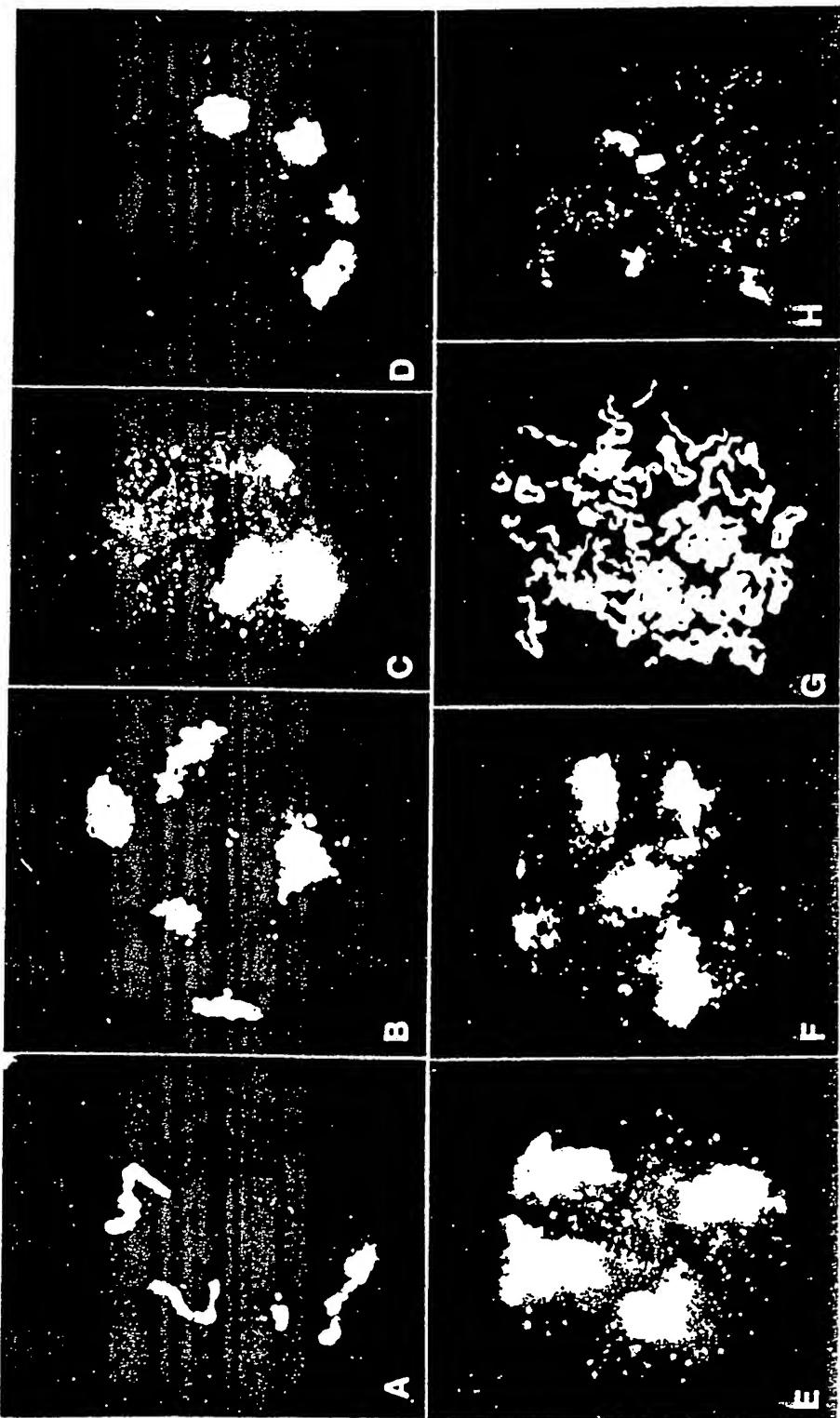
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FIG.9



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FIG.10



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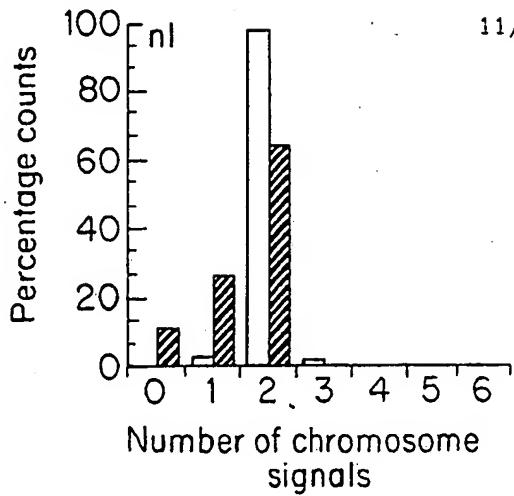


FIG. 11A

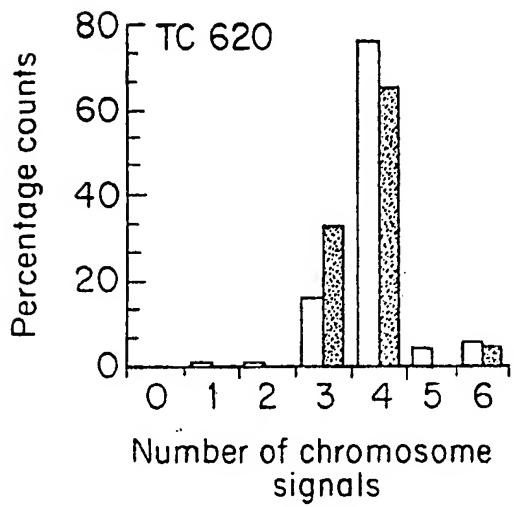


FIG. 11B

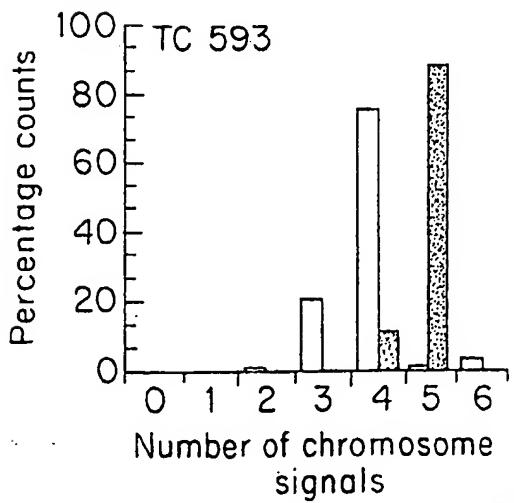


FIG. 11C

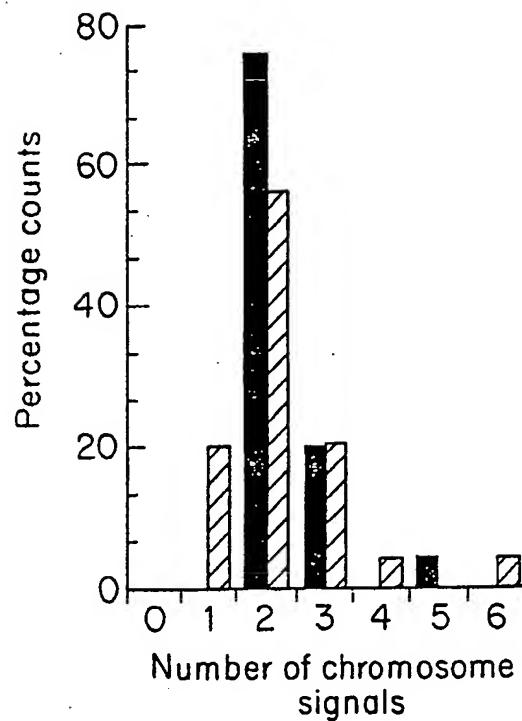


FIG. 11D

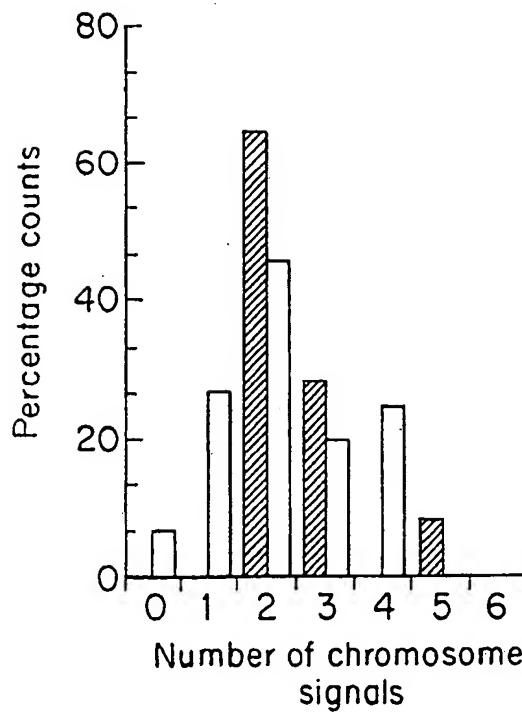


FIG. 11E

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FIG.12

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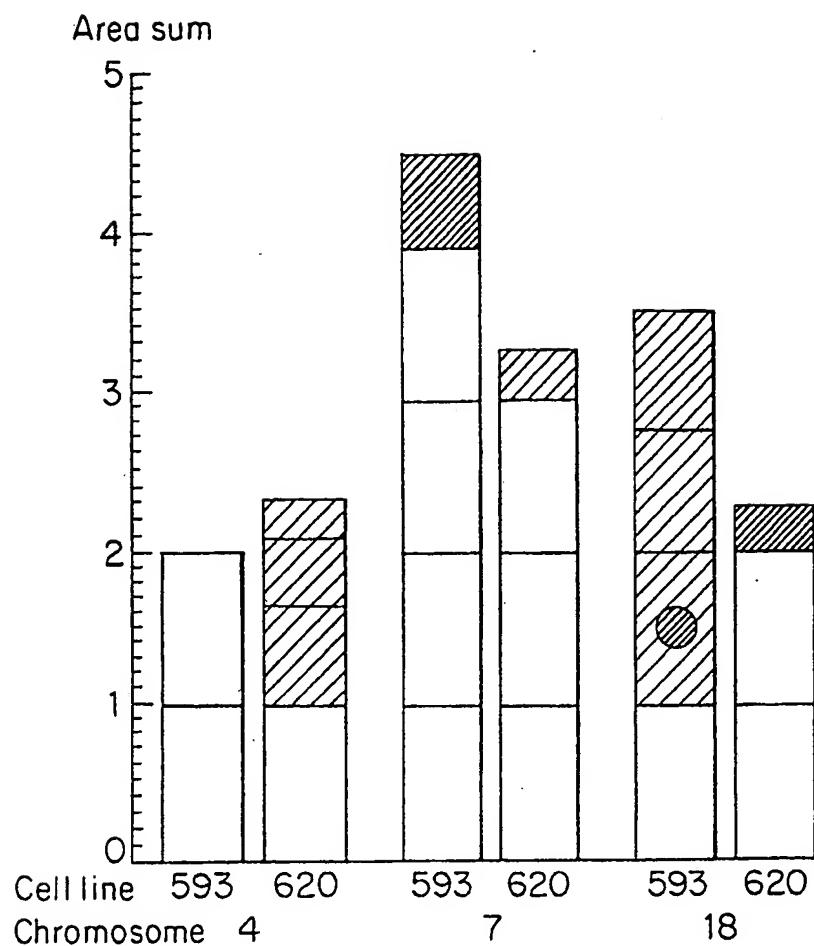
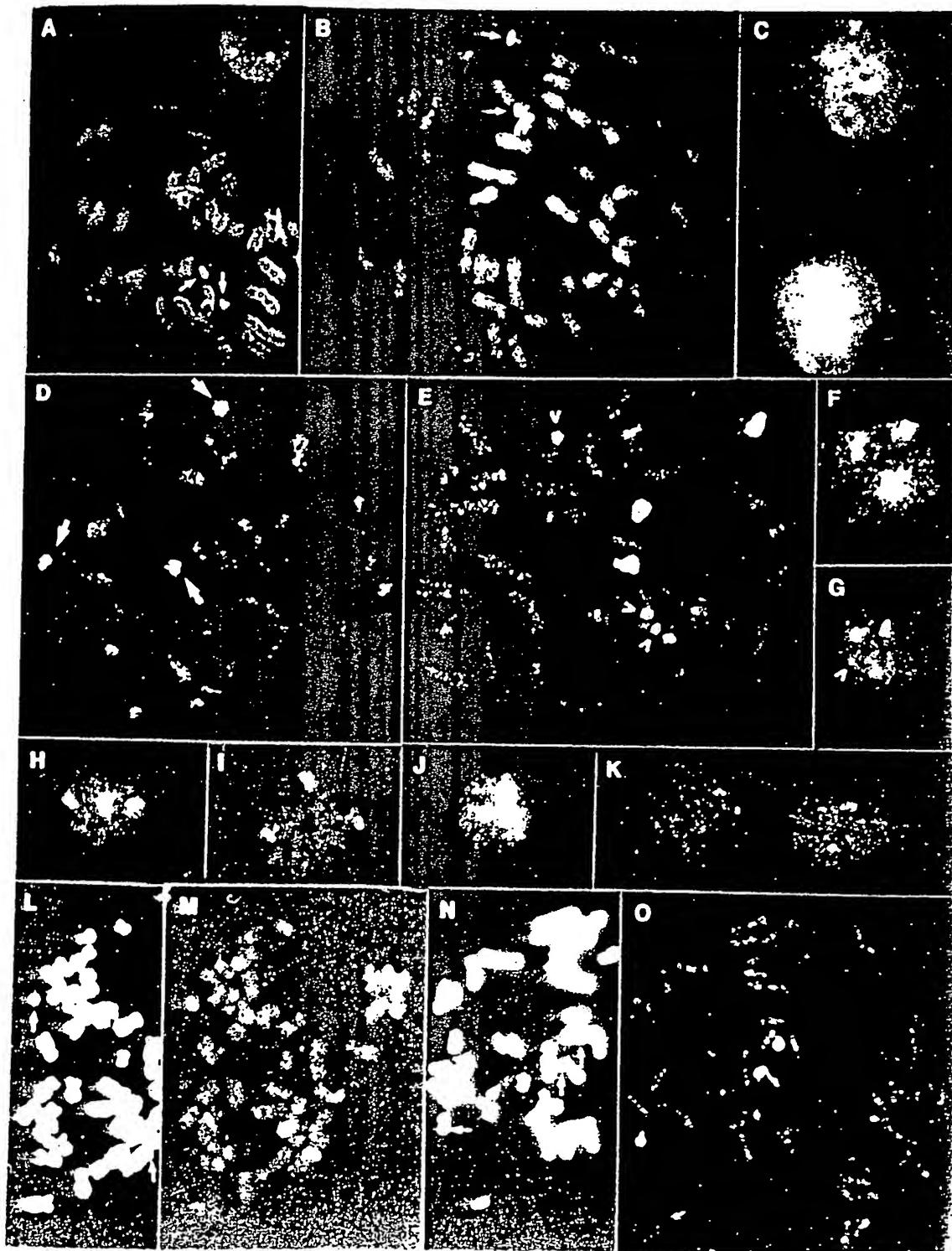


FIG. 13

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FIG.14



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INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 89/05253

I. CLASSIFICATION OF SUBJECT MATTER (Indicate classification symbols as you indicate all)

According to International Patent Classification (IPC), or to both National Classification and IPC

IPC5: C 12 Q 1/68

B. SEARCHED

SEARCHED

Cited in

IPC5

C 12 Q

III. DOCUMENTS CONSIDERED TO BE RELEVANT

Category "C" cited Document" "D" cited document, where appropriate, of the relevant "E" "F" "G" "H" "I" "J" "K" "L" "M" "N" "O" "P" "Q" "R" "S" "T" "U" "V" "W" "X" "Y" "Z" "Z1" "Z2" "Z3" "Z4" "Z5" "Z6" "Z7" "Z8" "Z9" "Z10" "Z11" "Z12" "Z13" "Z14" "Z15" "Z16" "Z17" "Z18" "Z19" "Z20" "Z21" "Z22" "Z23" "Z24" "Z25" "Z26" "Z27" "Z28" "Z29" "Z30" "Z31" "Z32" "Z33" "Z34" "Z35" "Z36" "Z37" "Z38" "Z39" "Z40" "Z41" "Z42" "Z43" "Z44" "Z45" "Z46" "Z47" "Z48" "Z49" "Z50" "Z51" "Z52" "Z53" "Z54" "Z55" "Z56" "Z57" "Z58" "Z59" "Z60" "Z61" "Z62" "Z63" "Z64" "Z65" "Z66" "Z67" "Z68" "Z69" "Z70" "Z71" "Z72" "Z73" "Z74" "Z75" "Z76" "Z77" "Z78" "Z79" "Z80" "Z81" "Z82" "Z83" "Z84" "Z85" "Z86" "Z87" "Z88" "Z89" "Z90" "Z91" "Z92" "Z93" "Z94" "Z95" "Z96" "Z97" "Z98" "Z99" "Z100" "Z101" "Z102" "Z103" "Z104" "Z105" "Z106" "Z107" "Z108" "Z109" "Z110" "Z111" "Z112" "Z113" "Z114" "Z115" "Z116" "Z117" "Z118" "Z119" "Z120" "Z121" "Z122" "Z123" "Z124" "Z125" "Z126" "Z127" "Z128" "Z129" "Z130" "Z131" "Z132" "Z133" 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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	Cancer Research, Vol. 48, 1988, Peter Devilee et al: "Detection of Chromosome Aneuploidy in Interphase Nuclei from Human Primary Breast Tumors Using Chromosome-specific Repetitive DNA Probes ", see page 5825 - page 5830 see especially page 5825	1-6,13-15
X	American journal of human genetics., Vol. 43, No. 3, 1988, D. Pinkel et al: "Detection of structural chromosome abberations in metaphase spreads and interphase nuclei by in situ hybridization high complexity probes which stain entire human chromosomes ", Abstract no. 0471 p. A 118	1-6,13-15
X	Experimental Cell Research, Vol. 176, 1988, T. Cremer et al: "Rapid Interphase and Metaphase Assessment of Specific Chromosomal Changes in Neuroectodermal Tumor Cells by in Situ Hybridization with Chemically Modified DNA Probes ", see page 199 - page 220 see especially p 200 line 42 - p 202 line 45	1-6,13-15
X	Hum Genet; Vol. 74, 1986, T. Cremer et al: "Detection of chromosome aberrations in the human interphase nucleus by visualization of specific target DNAs with radioactive and non-radioactive in situ hybridization techniques: diagnosis of trisomy 18 with probe L1.84 ", see page 346 - page 352 see especially p 346 col. 2 line 13 - p 348 col. 1 line 4	1-6,13-15

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. PCT/US 89/05253

SA 32708

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
GB-A- 2215724	27/09/89	FR-A-	2627192	18/08/89
		DE-A-	3904270	24/08/89
WO-A1- 87/05027	27/08/87	EP-A-	0261172	30/03/88
		JP-T-	63503272	02/12/88
WO-A1- 88/01300	25/02/88	ZA-A-	8705917	15/02/88